A NOVEL APPROACH FOR PREDICTING SUBLETHAL EFFECTS OF TOXICANTS TO AQUATIC ORGANISMS

FINAL SCIENTIFIC REPORT

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This study compared the effects of the water soluble fraction (WSF) of petroleum derived (P) JP-4, a common military and civilian jet fuel, and shale derived (SD) JP-4 on survival, growth, ventilatory rate, preference-avoidance behavior, and tissue of the bluegill sunfish (Lepomis macrochirus) to determine possible interrelationships and to determine which procedures might be most descriptive of sublethal stress. Comparative studies were also run using invertebrates and microbial communities. The dynamic 96-h LC50 for bluegill sunfish exposed to WSF P JP-4 was 26.2% (percent of the maximum soluble amount of JP-4). The dynamic 96-h LC50 for WSF SD JP-4 was 23.3%. In 21-d exposures to WSF P JP-4, no significant effects were observed on survival, growth, or behavior in concentrations ≤0.42%. Exposures of 0.95% WSF SD JP-4 for 21 d had no effects on survival or growth but appeared to be neurotoxic to fish, causing a tremor and ceasation

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of feeding for a period of 4 d. All effected fish recovered before the termination of the test. A concentration of 5.1% WSF P JP-4 caused a detectable shift in ventilatory rate and amplitude; this concentration was equivalent to the 96-h LC01. There was a significant shift in ventilatory rate at concentrations above 1.5% WSF SD JP-4 and a shift in ventilatory amplitude at 9.5%. Fish did not display a strong preference-avoidance reaction when exposed to WSF P JP-4. Some avoidance occurred at 3.5% and 4.9% WSF P JP-4. At concentrations near the 96-h LC50, fish appeared to lose their ability to detect WSF P JP-4, indicating that fish were unable to avoid lethal levels of WSF P JP-4. Bluegill had essentially no avoidance reaction to WSF SD JP-4. Bluegill had essentially no avoidance reaction to WSF SD JP-4. Few significant changes in whole and serum blood parameters were measured in fish exposed to 13% WSF P JP-4. At near lethal concentrations, changes were primarily attributed to osmoregulatory failure. Therefore such changes in blood parameters would not be useful in validating or confirming exposure to WSF P JP-4. Changes in blood parameters in response to WSF SD JP-4 exposures were less clearly explained. Liver damage was apparent, but osmoregulatory failure was not as definite as with the P JP-4. There were few significant changes in gill and liver histology of fish exposed to WSFs other than an increase in size or number of lipid vacuoles in the liver of exposed fish.

Only one LC50 could be determined for invertebrates in flow-through, acute toxicity tests with WSFs of both jet fuels. The 48-h LC50 for the dipteran, Paratanytarsus parthenogenica, exposed to WSF P JP-4 was 2.2%. The WSF SD JP-4 was not acutely toxic to this organism in concentrations ≤17.4%. Little or no mortality occurred in other acute tests with the oligochaete, Aeolosoma headleyi, and the cladoceran, Daphnia pulex. Jet fuel WSFs had no discernible effects on microbial communities in 48 h exposures. Chronic effect levels (lowest observable effect concentrations, LOECs) for invertebrates exposed to WSF P JP-4 ranged from 0.3 to 8.9%. Microbial communities showed increases in Autotrophic Index (AI) at exposures of 0.09 WSF P JP-4. Chronic sensitivity to WSF SD JP-4 ranged from 1.1 to >26.3%. Microbial communities showed increases in AI at 0.20% WSF SD JP-4.

In acute tests, fish were generally more sensitive to jet fuel WSFs than invertebrates. This is consistent with previous observations on the relative toxicity of the major components, benzene and toluene. No major discrepancies occurred in the chronic sensitivities of fish and invertebrates Fish ventilatory response appeared to be the quickest and most efficient of the sublethal tests used and provided a reasonable estimate of a chronic effect level. Microbial communities responded to low levels of jet fuel exposure, but toxicant related effects would probably be short-lived and of limited consequences in field exposures.

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PART I

I. INTRODUCTION

This is the third and final annual report for AFOSR Grant 82-0059. It is divided into four major sections. Part I is an overall introduction to the entire report with an abstract. Part II deals with the effects of the water souble fraction (WSF) of petroleum derived (P) and shale derived (SD) JP-4 of the bluegill sunfish (Lepomis macrochirus Raf.). Effects of WSF P and SD JP-4 on invertebrates are discussed in Part III, and Part IV is the administrative section of the report.

II. ABSTRACT

This study compared the effects of the water soluble fraction (WSF) of petroleum derived (P) JP-4, a common military and civilian jet fuel, and shale derived (SD) JP-4 on survival, growth, ventilatory rate, preference-avoidance behavior, and tissue of the bluegill sunfish (Lepomis macrochirus) to determine possible interrelationships and to determine which procedures might be most descriptive of sublethal stress. Comparative studies were also run using invertebrates and microbial communities. The dynamic 96-h LC50 for bluegill sunfish exposed to WSF P JP-4 was 26.2%

(percent of the maximum soluble amount of JP-4). The dynamic 96-h LC50 for WSF SD JP-4 was 23.3%. In 21-d exposures to WSF P JP-4, no significant effects were observed on survival, growth, or behavior in concentrations ≤0.42%. Exposures of 0.95% WSF SD JP-4 for 21 d had no effects on survival or growth but appeared to be neurotoxic to fish, causing a tremor and ceasation of feeding for a period of 4 d. All effected fish recovered before the termination of the test. A concentration of 5.1% WSF P JP-4 caused a detectable shift in ventilatory rate and amplitude; this concentration was equivalent to the 96-h LC01. There was a significant shift in ventilatory rate at concentrations above 1.5% WSF SD JP-4 and a shift in ventilatory amplitude at 9.7%. Fish did not display a strong preference-avoidance reaction when exposed to WSF P JP-4. Some avoidance occurred at 3.5% and 4.9% WSF P JP-4. At concentrations near the 96-h LC50, fish appeared to lose their ability to detect WSF P JP-4, indicating that fish were unable to avoid lethal levels of WSF P JP-4. Bluegill had essentially no avoidance reaction to WSF SD JP-4. Few significant changes in whole and serum blood parameters were measured in fish exposed to 13% WSF P JP-4. At near lethal concentrations, changes were primarily attributed to osmoregulatory failure. Therefore such changes in blood parameters would not be useful in validating or confirming exposure to WSF P JP-4. Changes in blood parameters in response to WSF SD JP-4 exposures were less clearly

explained. Liver damage was apparent, but osmoregulatory failure was not as definite as with the P JP-4. There were few significant changes in gill and liver histology of fish exposed to WSFs other than an increase in size or number of lipid vacuoles in the liver of exposed fish.

Only one LC50 could be determined for invertebrates in flow-through, acute toxicity tests with WSFs of both jet fuels. The 48-h LC50 for the dipteran, Paratanytarsus parthenogenica, exposed to WSF P JP-4 was 2.2%. The WSF SD JP-4 was not acutely toxic to this organism in concentrations ≤17.4%. Little or no mortality occurred in other acute tests with the oligochaete, Aeolosoma headleyi, and the cladoceran, Daphnia pulex. Jet fuel WSFs had no discernible effects on microbial communites in 48 h exposures. Chronic effect levels (lowest observable effect concentrations, LOECs) for invertebrates exposed to WSF P JP-4 ranged from 0.3 to 8.9%. Microbial communities showed increases in Autotrophic Index (AI) at exposures of 0.09% WSF P JP-4. Chronic sensitivity to WSF SD JP-4 ranged from 1.1 to >26.3%. Microbial communities showed increases in AI at 0.20% WSF SD JP-4.

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PART II

I. INTRODUCTION

A. Contents

This is Part II of the third and final annual report for AFOSR Grant 82-0059 and deals with the sublethal effects of the water soluble fraction (WSF) of petroleum derived (P) and shale derived (SD) JP-4 jet fuel on the bluegill sunfish (Lepomis macrochirus).

An abstract of research results is found in Part I of this report.

B. Background

Jet fuels such as JP-4 are used by both commercial and military aircraft. There is always potential for environmental exposure to fuels from leakage during transport, fueling operations, and mid-air dumping by aircraft making emergency landings. Once this fuel is inadvertently released into the environment, it can either directly or indirectly affect aquatic ecosystems. Potential lethal and sublethal effects of jet fuel can then be of great environmental concern.

Several studies have investigated the lethal and sublethal effects of jet fuel and its WSF on different species of fish (Cooper et al. 1981; Jenkins et al. 1977; Latendresse and Fisher 1983). Unfortunately, there have been very few dynamic (constant concentration) studies.

Using fish as biomonitors in determining the effects of pollutants on the aquatic environment is an accepted methodology. A recent review (Cairns and van der Schalie 1980) found relatively few biomonitoring techniques in practical use today--perhaps due to cost and lack of legally mandated requirements. They also noted that direct measurement of sublethal responses is a valid methodology for extrapolating information on the no observable effect concentration (NOEC) for hazard evaluation. Collection of quantitative sublethal information is increasingly necessary in determining true effects of pollutants on organisms and ecological systems. Collection of such data with computer assisted equipment generates information bases that are statistically sound (Cairns 1981).

A computer assisted biomonitoring technique successfully used to illustrate sublethal effects of pollutants using respiratory or ventilatory movements was developed by Cairns et al. (1970). Measurement of respiratory movements was shown to be a valid measurement of actual ventilatory rates (Heath 1972). Ventilatory measurement techniques were used

by Thomas and Rice (1979) to demonstrate effects of crude oil on ventilatory rates in salmon.

Another biomonitoring procedure used to investigate effects of sublethal levels of suspected toxicants is preference-avoidance behavior (Cherry and Cairns 1982). Determination of whether fish will actively avoid pollutants is an important consideration in evaluating their potential environmental impact, i.e. death of organisms, loss of habitat, etc. Such studies have been conducted by Weber et al. (1981) using petroleum hydrocarbons in in-situ experimentation. These studies found differences in behavior that were related to age of salmon. Juvenile salmon were more sensitive to petroleum hydrocarbon contamination when migrating downstream than were adult salmon that were migrating upstream to spawn.

Physiological measurements are useful in establishing effects of toxicants on organisms. Human blood parameters have been studied for many years and evaluation of poikilotherm blood was recommended over three decades ago (Smith et al. 1952). Many problems have occurred with blood studies since insufficient background data are available for "normal" levels of blood constituents for most species of fish; consequently, determining significant deviations from usual levels or the meaning of such changes is difficult (Warner et al. 1979). The concept of using specific enzymes

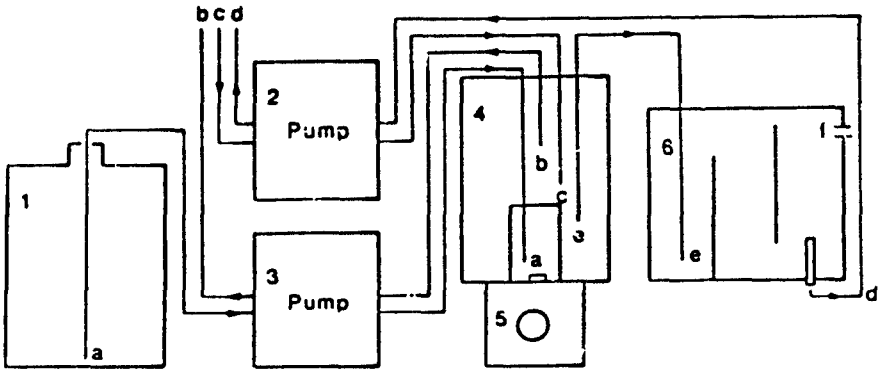
as indicators of petroleum pollution has been used with mixed results by Payne and Penrose (1978) and Rutherford et al. (1979).

No comparative, simultaneous studies have used a series of these state-of-the-art sublethal evaluative techniques on one toxicant for comparing results and determining which techniques might be most useful and descriptive of problems occurring in effected organisms. This research, which uses petroleum derived and shale derived JP-4 jet fuels, attempts to fill this gap.

II. MATERIALS AND METHODS

A. Generation and Analysis of WSF JP-4

The JP-4 fuels for this research were obtained from the Fuels Laboratory, Wright-Patterson AFB, OH. A flow-through system similar to that of Dauble et al. (1981) was constructed to generate WSFs. The system consisted of a mixing chamber, settling chamber and accompanying pumps for jet fuel and water (Figure 1). The mixing chamber consisted of a glass battery jar with a smaller beaker attached with silicon in the center. The battery jar was set on a magnetic stirrer with the stirring bar in the inner beaker. Jet fuel and carbon dechlorinated tap water were pumped into



WATER SOLUBLE FRACTIONATOR

Figure 1: Water soluble fractionator used for exposing bluegill. Jet fuel flows from tank 1 through line a via pump 3 to mixing chamber 4. Water flows through line c via pump 2 to chamber 4. The WSF flows from chamber 4 to settling chamber 6 through line e and from there through line d to research equipment via pump 2. Excess fuel is removed from 4 through line b via pump 3. Excess WSF exits through line f to waste.

the chamber at approximately a 5% vol:vol ratio. Floating ("spent") JP-4 was removed from the mixing chamber at a rate equivalent to fresh JP-4 delivery. Air from an aquarium pump was injected into the beaker to aid in mixing and oxygenation of the mixture. The WSF JP-4 flowed from the mixing chamber to the settling chamber, a rectangular glass box with baffles, where any entrained jet fuel was separated. The mixing and settling times were ca. 1 h, depending on flow rates of fuel and water. The fractionator was capable of delivering 150 ml min^{-1} WSF JP-4. The WSF JP-4 was either pumped or allowed to flow by gravity to the experimental equipment. This fractionator was used for all experiments except for preference-avoidance studies where higher flow rates required a larger volume batch system. The batch system used two 55-gal drums in which a 5% JP-4 solution was mixed for 6 to 12 h. The mixture was allowed to settle for at least 6 h. The initial WSF concentration was determined, and the solution was used in the experiments.

Toluene and benzene, the largest and most volatile components of the WSFs, were used as markers to calculate WSF concentration (Figure 2). The assumption was made that since these markers were the most volatile fractions of the WSFs then if they were present in significant concentrations there was no significant loss of other components. These more volatile components are also the most toxic (e.g.,

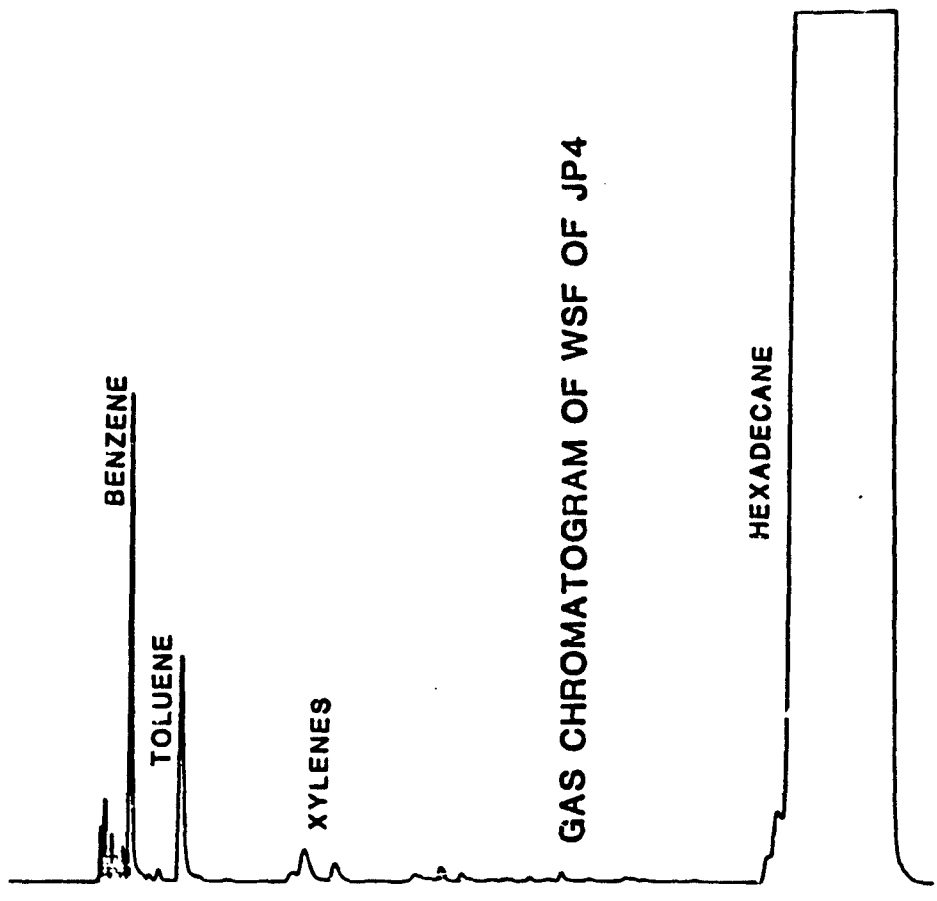


Figure 2: Typical chromatograph for saturated solution of WSF JP-4.

Morrow et al. 1975). The WSF concentrations were defined as per cent of maximal WSF (MaxWSF), based on height of toluene and benzene peaks relative to a standard. The standard MaxWSF was defined as that concentration resulting from constant speed mixing of a 5% JP-4 and carbon dechlorinated tap water sample for 3 h at 25°C. This mixture settled for 3 h before extraction.

Procedures modified from Fisher et al. (1984) were followed in analyzing benzene and toluene markers. A 100-ml water/WSF sample was collected in a volumetric flask, and 1 ml of hexadecane was added. The flask was vigorously shaken for 3 min and allowed to settle for another 3 min. A 6-ul subsample of the separated hexadecane layer was analysed by gas chromatography.

A Varian 1600 gas chromatograph with a 3-m SE30 column and flame ionization detection was used to analyze WSF JP-4. The chromatograph was programmed to maintain 40°C for 5 min and then increase by 10°C min^{-1} until a final temperature of 200°C was reached. The injector was set at 220°C and the detector at 225°C. Helium, the carrier gas, was delivered at 20 ml min^{-1}; hydrogen and air for the flame were delivered at 30 ml^{-1} and 300 ml^{-1}, respectively.

Standards were made for toluene and benzene as well as several other known constituents of WSF JP-4 (e.g., three

different xylene isomers and napthalene) using pure chemicals obtained from Fisher Scientific. Standards were used to determine the detention time of each constituent in the GC column and relative concentrations in WSF P JP-4.

B. Experimental Organisms

Juvenile bluegill sunfish obtained from the Kurtz Fish Hatchery in Elverson, PA, were 4.90 cm (standard deviation (SD), SD±0.20) in standard length (SL) and 3.53 g (SD±0.44) in weight. These fish were used for the acute toxicity and preference-avoidance testing.

Juvenile bluegill from an alternate commercial source, Perry Minnow Farm in Suffolk, VA, were used for 21-d exposures. Mean length of these fish was 3.49 cm (SD±1.20), and mean weight was 2.53 g (SD±0.26)

Adult bluegill, required to provide sufficient blood for analysis, were seined from a small, closed pond on the grounds of the Veterans Administration Hospital in Salem, VA. Mean SL of these fish was 11.18 cm (SD±0.48) and mean weight was 42.93 g (SD±5.77). Smaller adults (ca. 5.0 cm SL) were also seined from the hospital pond for use in ventilatory studies. All fish were placed in laboratory holding tanks, prophylactically treated with tetracycline, and held for at least 2 wk before use.

Lethality tests verified the hypothesis that no significant differences occurred in the acute responses of two fish populations and three age groups. (See Part II, Section III.B.)

Carbon dechlorinated tap water was used throughout this research for holding all test organisms and as a diluent for WSFs. Mean pH was 8.17 (SD±0.01; range, 7.6 to 8.7); mean hardness was 58.72 ppm (as $CaCO_3$, SD±10.88); mean alkalinity was 36.02 ppm (as $CaCO_3$, SD±8.70); and mean conductivity was 1.193 mv (SD±0.11).

C. Toxicity of JP-4 Jet Fuel

Static acute toxicity tests were run in 3-l glass aquaria with 10 fish in each exposure container. These static toxicity tests were run using JP-4 layered directly onto the water surface. (These were defined as being "neat" exposures as opposed to using the WSF of the JP-4.) These toxicity tests were run to determine the effect of a solitary, one time spill of JP-4 jet fuel in a static pond-type of environment where very little mixing would occur. A series of concentrations of JP-4, from 1 to 100 parts per thousand (ppt), was used for range-finding toxicity tests. The actual static tests were run with 1, 2, 3, 5, 8 ,and 10 ppt. They were conducted as 96-h tests with observations for lethality made at 15 min, and at 0.5, 1, 6,

and every 24 h thereafter. The pH and dissolved oxygen in each tank were also determined daily.

Dynamic 96-h flow-through toxicity tests were conducted using a modified Mount-Brungs dilutor system made of glass and teflon (Figure 3). These studies were performed to model what might happen in a situation where there was a continual discharge of jet fuel in an environmental system, such as a stream, where mixing would occur. This was considered the most realistic way to evaluate lethality as mixing would occur, at least from wind action, in most natural environments. To avoid WSF loss due to excess head space, toxicant chambers were sized and covered to reduce air space. Diluent and WSF mixing occurred in a closed system using glass Y tubes. The dynamic test was repeated three times with 10 fish per each of six concentrations. Observations of fish lethality, pH, and dissolved oxygen were made on the same time basis discussed previously for the static tests. Results were analyzed using log-probit (Finney 1976) and Spearman-Karber (Hamilton et al. 1977) procedures.

Juvenile bluegills were also exposed to sublethal concentrations of WSFs for 21 d in order to evaluate effects on survival, growth, and behavior in longer, chronic exposures. On the day of the test, 10 fish were randomly distributed to eight groups, one at a time. Two groups of

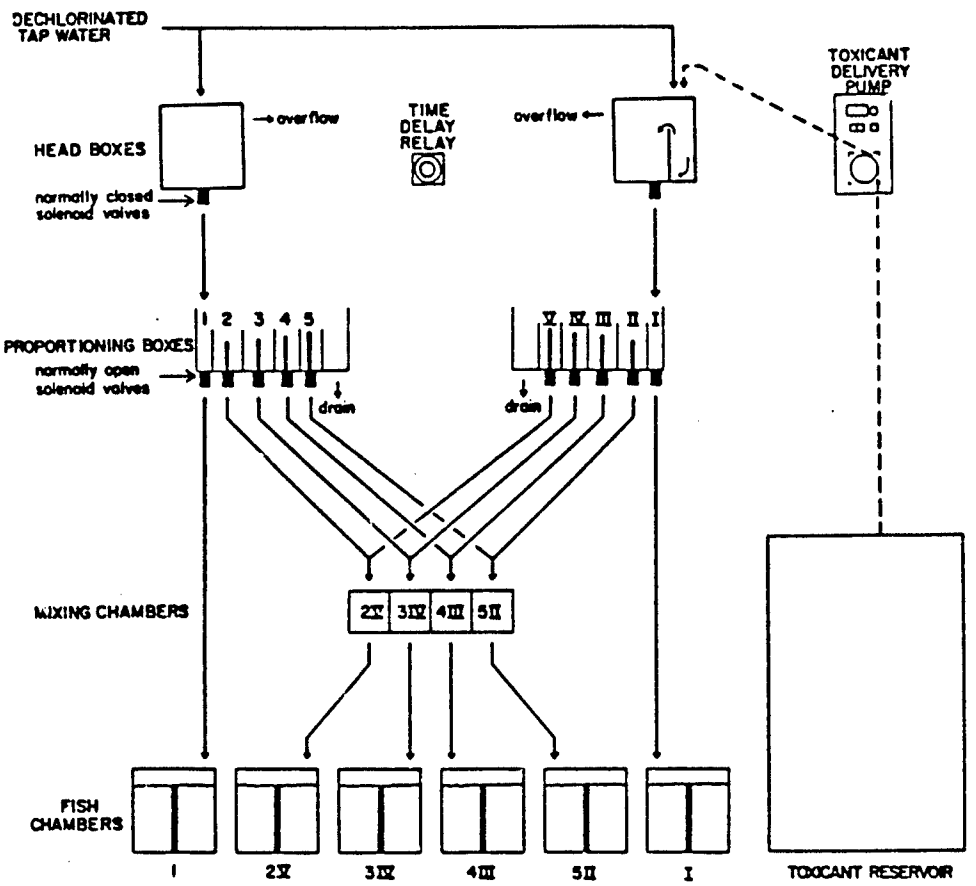


Figure 3: Dynamic diluter system used for acute toxicity studies.

fish were immediately sacrificed, and initial lengths and weights were determined. Mean weight and length of the two groups were compared using t-tests in order to determine differences in groups of fish before exposure to WSFs. The remaining groups of fish were randomly assigned to five treatment levels and a control. The highest dose was approximately half the dynamic LC50. Subsequent doses followed a 50% dilution series. Exposures were conducted in the Mount-Brungs dilutor system (Figure 3). Fish were fed Tetramin fish food ad libitum daily. Survival and behavior of fish, dissolved oxygen concentration, and temperature of test water were noted daily. Hardness, alkalinity, pH, and conductivity of dilution water were determined weekly. After a 21-d exposure, all surviving fish were sacrificed and measured. Differences between exposure concentrations in length and weight were examined with ANOVA and Duncan's multiple range test.

D. Ventilatory Rate Studies

Ventilatory studies were conducted on bluegill using a system developed by Cairns and co-workers (e.g., Cairns and Thompson 1980; Thompson et al. 1983). Bluegill (SL ca. 5.0 cm) were randomly placed in small plexiglass chambers (700 ml) with front and rear electrodes (Figure 4). After placement in the chambers, fish were allowed to acclimate for 2 d before background ventilatory rates were determined.

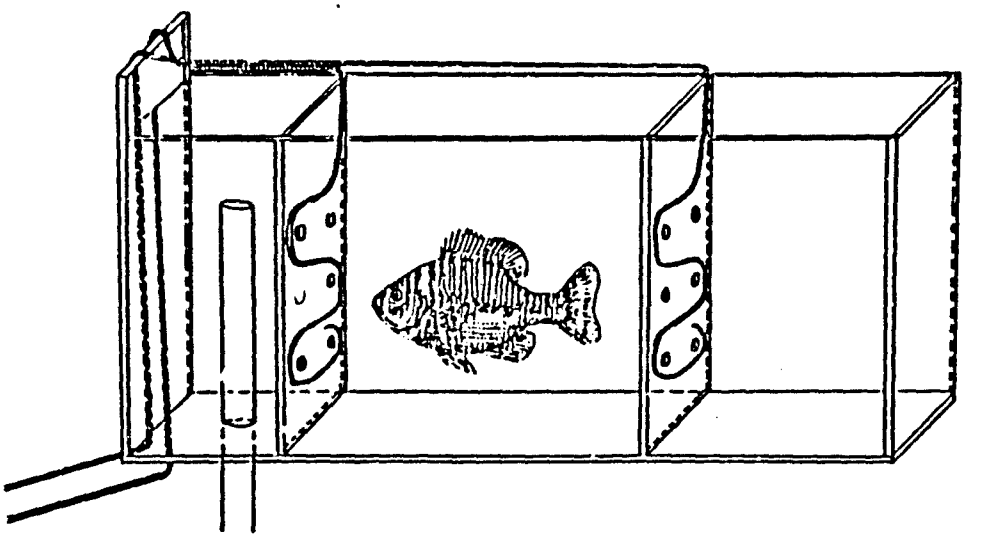


Figure 4: Detail of experimental chamber used to measure ventilation of bluegill.

Approximately half the fish were subsequently exposed to WSF JP-4; others served as controls, and ventilatory data were collected for another 24 h. Fish chambers were located in a closed container with constant light from three 15-watt bulbs. The container was buffered from vibration in the laboratory by anchoring its supports in sand; eliminating much extraneous stimuli (Figure 5). Fish movement was detected as a small voltage change (ca. 50 mv) by the electrodes; this was then amplified and converted from analog to digital format by existing hardware and software for a Digital Equipment Corporation PDP8/E computer (Thompson et al. 1978). A ventilatory count was defined as any electric pulse that had both a positive and a negative peak in excess of a threshold of one volt after amplification. Gruber et al. (1979) have shown that each of the pulses corresponds to one full ventilatory cycle of a fish. The computer assisted program counted ventilatory rates and averaged the amplitudes of opercular beats over 15-min periods. This information was then stored on a magnetic tape by a Decassette TU60 cassette tape drive and printed by a Decwriter II (LA36).

After data collection was completed, the minicomputer was tied into the Virginia Polytechnic Institute and State University's (VPI&SU) main frame computer system running VM/CMS on a IBM370-1580. Data were then transferred directly to the Conversational Monitoring System (CMS) for

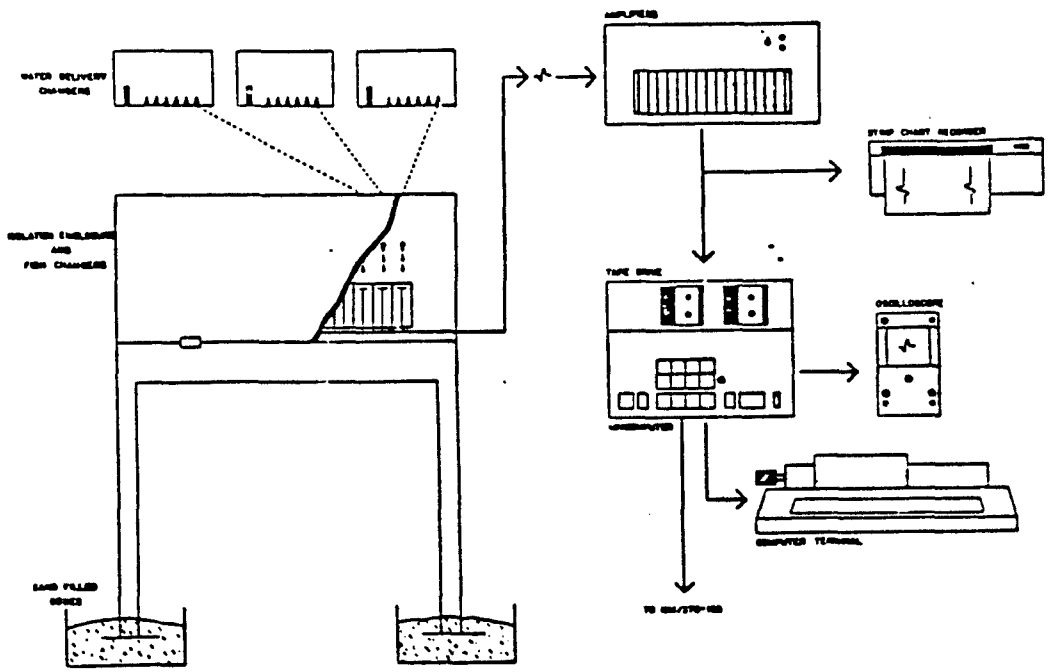


Figure 5: Experimental setup for ventilatory studies.

later statistical analysis. Data were analyzed with ANOVA or with Wilcoxon rank sum procedures if the Kolmogorov D statistic suggested a non-normal distribution.

E. Preference-Avoidance Studies

Fish were given a side-by-side challenge of clean (carbon dechlorinated tap water) and WSF influenced water in preference-avoidance studies. A system slightly modified from that of Lubinski et al. (1977) was used (Figure 6). The exposure chamber was located within an isolation box, and fish behavior was observed via closed circuit TV so that fish were subjected to as little extraneous stimuli as possible. The concentration of WSF JP-4 was determined from samples collected from ports at the bottom of each side of the chamber.

Three different observational techniques were used to determine fish behavior in the preference-avoidance studies:

(1) Fish were visually observed directly on a closed circuit TV system. Five fish were placed in the exposure chamber for each study and their positions manually recorded every 30 sec.

(2) On alternate 30-sec periods, the number of fish movements was visually quantified by dividing the TV screen into quadrants (Figure 7). A movement was defined as any time any fish crossed any quadrant line.

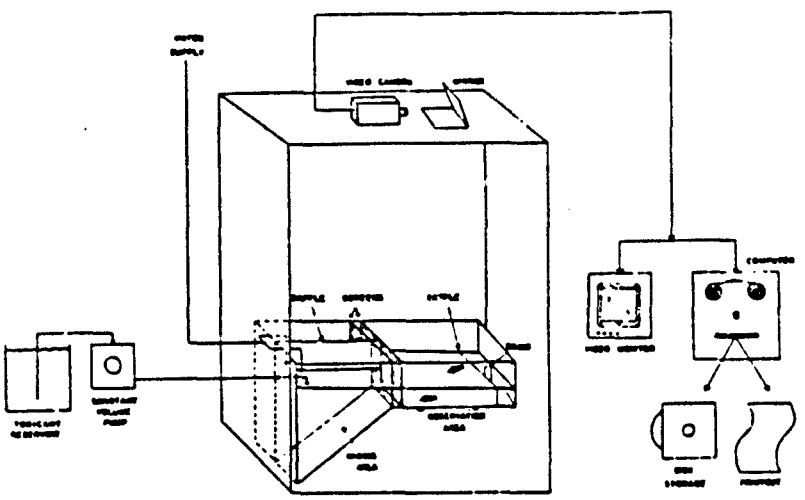


Figure 6: Experimental equipment for preference-avoidance studies with bluegill.

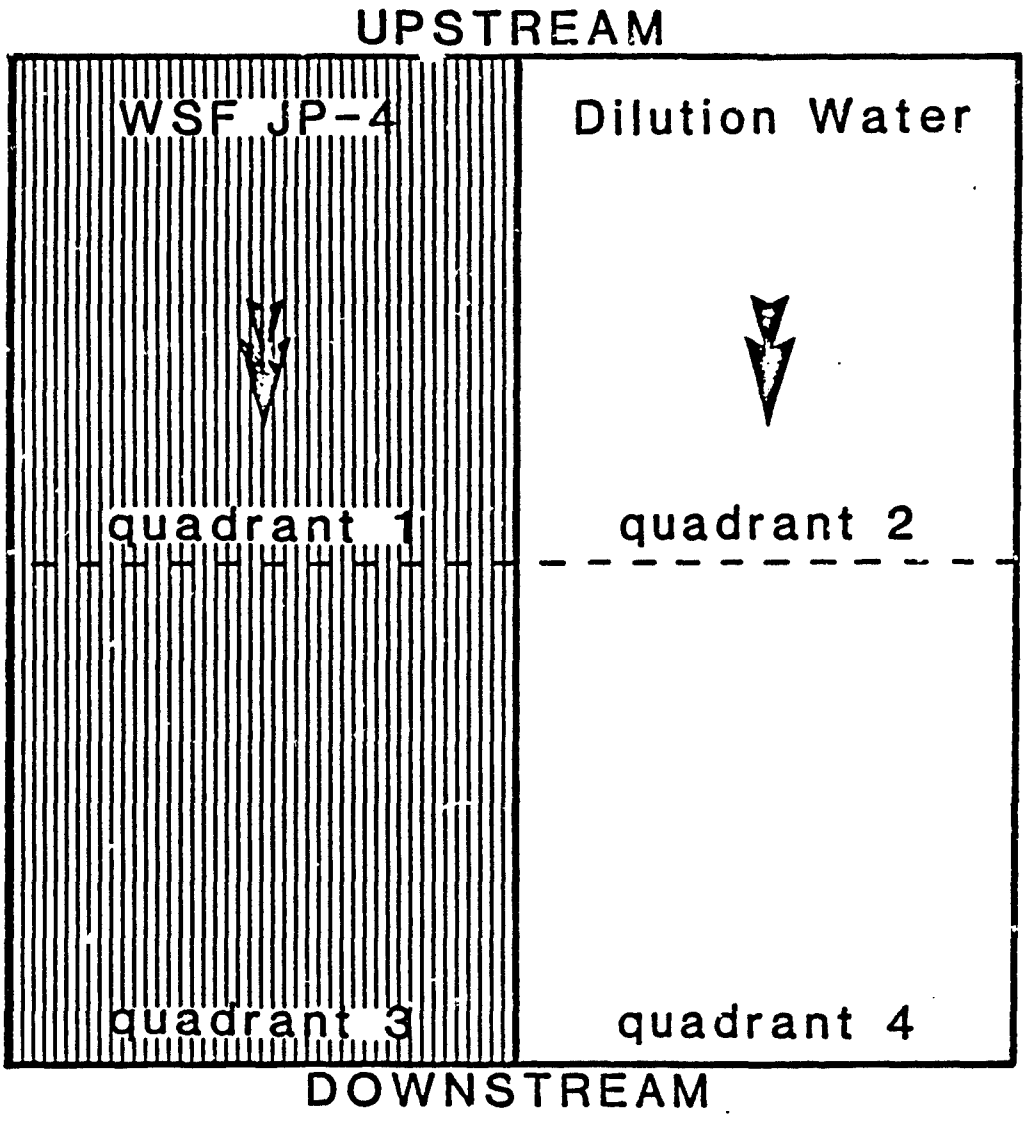


Figure 7: Example of quadrant grid for monitoring movement of bluegill exposed to WSF JP-4.

(3) Measurements of time fish spent on either side of the tank, dilution water only side vs WSF influenced side, were also made every 30 sec using a programmed Cromenco Z-2 computer (an 8080-based microcomputer). All fish were seen by the system as blocks of dark "pixels" on the light TV screen. The number of blocks of dark pixels for each quadrant were totaled every 30 sec. This information was then stored on floppy discs for later retrieval and transfer to VPI&SU's IBM 370-1580 for statistical analysis. This technique was used to confirm the results obtained visually; visual observations were used for all data reported in Part II, Section III.D.

Fish were removed from holding tanks and placed in the preference-avoidance chamber to acclimate for 1 to 2 h prior to exposure to WSFs. Two separate 6-h control runs were carried out to verify that there was no preference by fish for any section of the exposure chamber. In the first exposure study for WSF P JP-4, three 10-min observations of behavior were made at control (0% WSF) level. The WSF P JP-4 was then pumped to one side, and, after allowing 5 min for the concentration to come to steady state, behavioral observations were again made. A sample was taken from the exposure side and the concentration of WSF P JP-4 was increased to the next higher level. This study was repeated eight times, alternating the exposure side for each study; it is shown as Study 1 in Figure 8.

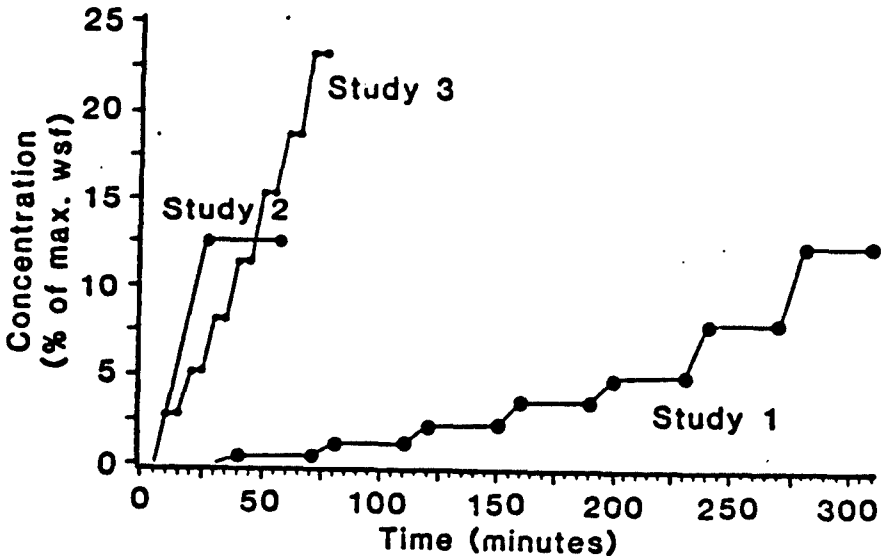


Figure 8: Time-dose exposures used in preference-avoidance studies.

After results of the first study indicated possible acclimation of fish to WSF P JP-4, another study was conducted in which the concentration of WSF P JP-4 was increased directly from 0% WSF P JP-4 to the highest level tested in Study 1. This study was conducted twice, alternating the exposure side; it is shown as Study 2 in Figure 8.

In the last study for WSF P JP-4, fish were exposed to more rapid increases and higher final concentrations of WSF P JP-4. Due to requirements for high flow rates, only one 7-min observation of behavior was made at each concentration in each replicated study. This study was repeated eight times, again alternating exposure sides; it is shown as Study 3 in Figure 8.

The above data were analyzed using ANOVA to determine if there were any significant differences. If differences were found, an LSD procedure was used to determine where the differences were.

For the studies with the WSF SD JP-4, a slightly different exposure procedure was used. The exposure period for each concentration was 7 min, and 3 readings were taken at each concentration. This study with WSF SD JP-4 was replicated eight times.

F. Blood Chemistry

Fish were exposed to two different concentrations of WSF P JP-4, 13% for 96 h and 26% for 24 h, to evaluate their effect on blood chemistry parameters. These exposures were ca. 50 and 100% of the 96-h LC50. Exposed and control fish were bled after anesthetization in a 5% benzocaine bath. The caudal peduncle was excised and blood removed from the dorsal aorta using 1 cc needleless syringes. When possible, determinations were performed on blood from individual fish. In most cases blood from two or more fish was pooled into one sample for a sufficient volume to perform all desired analyses.

Blood parameters were divided into whole and serum blood chemistry tests. The whole blood tests consisted of hematocrit, hemoglobin concentration, and red blood cell (RBC) counts

(1) The hematocrit test, yielding percent total blood cells (e.g., RBC, white cells, platelets, etc.) in whole blood, was performed using standard heparin coated microhematocrit tubes. A tube was filled with blood directly from each sacrificed fish, and percent hematocrit was decermined with a Clay-Adams Hematocrit Reader after the tubes were spun down in a Clay-Adams Hematocrit Centrifuge for 10 min (Wintrobe 1974).

(2) The RBC counts were conducted by diluting whole blood 1:200 with Dacie's solution and placing a small volume of diluted blood on a hemacytometer. For each count, five ruled areas on the hemacytometer were enumerated, averaged and corrected to cells mm^{3-1} (Hessler 1960). The morphology of the RBCs was also checked at this time.

(3) Hemoglobin concentration, an indicator of oxygen carrying capacity, was measured on pooled samples of whole blood using the cyanmethemoglobin method (Wintrobe 1974).

Blood serum was analyzed using a Gilford 3500 computer assisted blood chemistry analyzer, applicable Gilford reagents, and appropriate technical instructions. Blood serum tests were conducted for

(1) Enzymes lactate dehydrogenase (LDH) (EC 1.1.1.27) and aspartate aminotransferase (SGOT) (EC 2.6.1.1);

(2) Inorganic substances calcium, chloride, magnesium, and inorganic phosphorous;

(3) Albumin and total protein concentration; and

(4) Glucose.

Several other techniques were also used to determine the physiological condition of fish:

(1) Two different types of commercially available reagent strips for human urinalysis were used to examine fish mucous components:

(a) Hemastix[1] were used to evaluate the amount of hemolysis that had taken place in fish blood (Smith and Ramos 1976). If significant hemolysis of RBCs occurred as a result of exposure to WSFs, detectable amounts of breakdown components should be evident in the mucous.

(b) Ketostix[1] evaluated ketone bodies in the mucous of fish (Ramos and Smith 1978). If fish were starving and breaking down fatty acids for subsequent metabolism, ketone bodies should be present in the blood and should also appear in the mucous.

(2) Tissue water content, an indicator of possible osmoregulatory problems, was measured on muscle and liver that were dissected from fish, weighed, dried and reweighed.

3) ATP levels, which can be an indication of sublethal stress (Kennicut 1980; Suresh et al. 1983; Heath 1984), in fish liver were determined with an enzymatic technique in which an ATP reaction with phosphoglycerate kinase was observed with a spectrophotometer (Jaworek et al. 1974).

The data were analyzed using a Wilcoxon rank sum procedure to determine significant differences as the data were not uniformally normally distributed.

Studies on WSF SD JP-4 were conducted slightly differently. One exposure concentration and two exposure time periods

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[1] Registered trademark of Miles Laboratory, Inc.

were studied. The concentration of WSF SD JP-4 used was ca. 22%. This value was very close to the 96-h LC50. This value was chosen because so few changes were observed at the lower concentration used for the WSF P JP-4. The two time periods were 24 h and 96 h.

G. Histology of Fish

The same fish that were exposed in the blood chemistry studies discussed previously were used for histology studies. Liver and gill tissues were removed from fish immediately after they had been bled. Tissues were fixed initially with gluteraldehyde and then with osmium tetroxide (Meek 1976). After fixation, tissues were dehydrated with a series of increasing concentrations of alcohol:water dilutions, ending with a 100% alcohol solution. After dehydration tissues were embedded in Spur's embedding media and then shaped and sectioned on a microtome. Thick sections (ca. 2.0 um) were cut for viewing with a Leitz Dialux light microscope with attached camera. Thin sections (ca. 60-90 nm) were cut for viewing with a JOEL-JEM 100C electron microscope.

Comparative studies were made of tissue from control and exposed fish using photomicrographs taken of both tissue sections. In addition to visual impressions of the general state of the tissues, electron micrographs of liver tissue

were evaluated for morphological differences with a planitmetry technique modified from that of Weibel (1969). Random micrographs of tissue were taken, and relative amounts of different cellular components were calculated and statistically compared. Electron micrographs were taken at 33,000x. The actual planitmetry method consisted of making 8 X 10 prints of the micrographs and overlaying graph paper on each print. Intersection of the major graph lines were then punched with a sharp point so that each electron micrograph was impressed with a grid of 54 holes (six columns of nine holes) spaced 2.54 cm apart. Several electron micrographs were punched at the same time, and, since the graph paper was opaque, the distribution of the holes on each electron micrograph was random; there was no a priori placement of the grid. Each hole was considered a positive count if it landed on one of the tissue organelles of interest, such as a vacuole, endoplasmic reticulum, mitochondria, a nucleus, or glycogen deposits. The numbers of positive counts were then compared using a t-test to determine if any significant differences in the relative counts of the different cellular materials occurred.

III. RESULTS AND DISCUSSION

A. Generation and Analysis of WSFs JP-4

The fractionator efficiently generated a reliably uniform concentration of WSFs during acute tests. Analysis of WSF P JP-4 showed the two primary components, benzene and toluene, to be present in MaxWSF at 16.4 ppm and 12.2 ppm respectively. These values agreed very well with those provided by the source of the P JP-4, the USAF Fuels Laboratory, Wright-Patterson AFB, OH. A complete chemical characterization was not attempted; such a characterization can be found in Smith et al. (1981).

One problem encountered with the fractionator was the growth of a microorganism capable of very rapid degradation of the soluble components of the WSF JP-4 during longer exposure periods. This was partially controlled by periodic dissembling of the apparatus, disinfection with chlorine, washing with soap and water, and copious rinsing with dechlorinated water.

B. Toxicity of JP-4 Jet Fuel

The static toxicity of the "neat" P JP-4 was calculated using a log-probit analysis (Finney 1976). The resulting 96-h LC50 was 1.74 ppt (95% fiducial limits (FL), FL = 1.27

to 2.13 ppt; slope = 7.5±2.13; X^2 = 0.72). Although this is reported as a 96-h result, realistically it was only a 24-h test due to the high volatility of the JP-4 components. Within the first 24-h period, concentrations of benzene and toluene were reduced by >50%.

The individual dynamic acute toxicity tests resulted in few partial kills so that the log-probit statistical analyses could not be performed on each repetition. A Spearman-Karber statistical analysis (Hamilton et al. 1977), which does not depend on number of partial kills was used to generate an LC50 for the dynamic toxicity tests (Rice et al. 1977). With this statistical technique, the calculated 96-h LC50 for WSF P JP-4 was 26.2% (95% FL = 24.8 to 27.7%) MaxWSF. This is approximately equal to 11.6 ppm of total soluble P JP-4 components (Fisher et al. 1984). In order to estimate the slope of the toxicity curve, data from all replicates of dynamic toxicity tests were combined for log-probit analysis. The result was a 96-h LC50 for WSF P JP-4 of 28.8% (95% FL = 26.0 to 33.4%; slope = 14.02±7.92; X^2 = 0.87). (These two 96-h LC50s were not significantly different as determined by overlap of confidence limits.) The advantage of performing log-probit analysis on all data although it is not a normally accepted procedure was that it illustrated the steepness of the dose-response curve. Virtually all lethality (99%) occurred between 19.5% and 38.8% WSF P JP-4 (Figure 9).

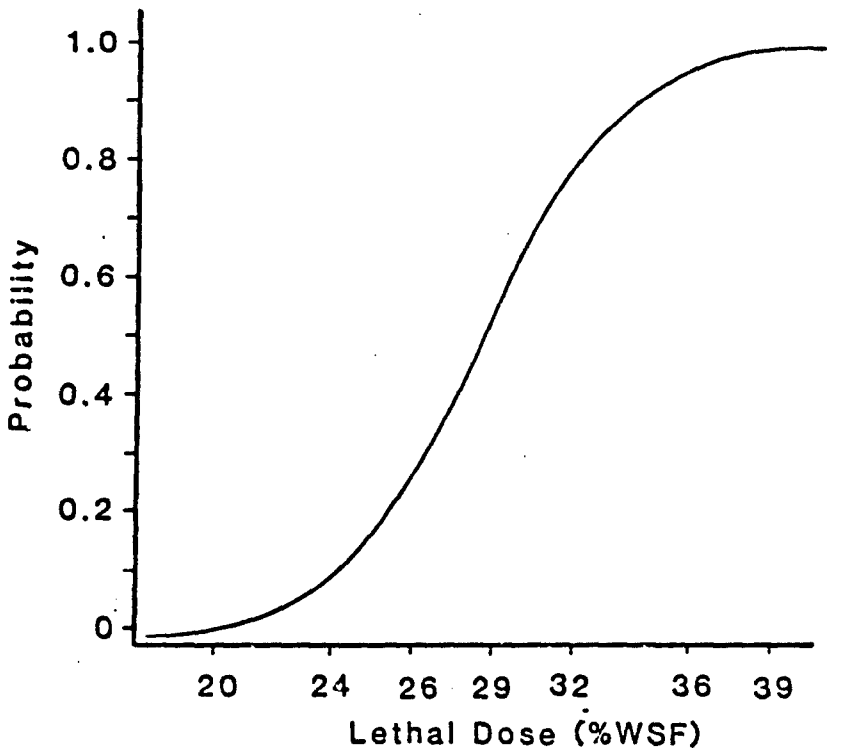


Figure 9: Dose-response acute toxicity curve for WSF JP-4.

The 24-h (25.5% WSF), 48-h (26.4% WSF) and 72-h (28.7% WSF) LC50s for WSF P JP-4 were also determined using log-probit aralysis of available data for each time period. Relative potency analysis showed that no significant difference occurred between these four values (95% confidence limit (CL), CL = 95%, Finney 1976). Apparently WSF P JP-4 is toxic to fish based on a threshold of toxicity rather than on increased time of exposure. There were few significant deaths of fish in the toxicity tests after the first 24 h. Although there were great losses of the more volatile components within 24 h, the 96-h LC50 reported by Fisher et al. (1984) for a similar fuel had an equivalent slope (9.9 to 12.8) and a higher LC50, 42 to 48% WSF P JP-4, which would be expected for a static test.

Acute toxicities of the main components of WSF P JP-4 to bluegill have been reported by other investigators. Benzene was found to have a 96-h LC50 of 22.49 mg/l (Pickering and Henderson 1966). In their review, Buikema and Hendricks (1980) quote values for the 24-h LC50 of 20 and 32 to 34 mg/l for benzene. The reported 96-h LC50s for toluene were 24.00 mg/l (Pickering and Henderson 1966) and 6.41 to 8.01 mg/l (Korn et al. 1979). The 24-h LC50s quoted by Buikema and Hendricks (1980) were 18.9 and 24 mg/l. As WSF P JP-4 is a composite of benzene, toluene, and other minor components, the LC50s for the major components make it appear that they act additively with the less concentrated

components of WSF P JP-4 in exerting their toxic effect. A calculated 96-h LC50 for WSF P JP-4 from the above data would be ca. 18 ppm. Since this calculation is based on static data, it compares well with the dynamic data for WSF P JP-4 (LC50 = 11.6 ppm), which would be expected to show a higher toxicity. This also supports the assertion that benzene and toluene are the more toxic components of petroleum products (Craddock 1977; Morrow et al. 1975). The data for WSF P JP-4 is also comparable to that presented by Thomas and Rice (1979) for the WSFs of Cook Inlet Crude (LC50 = 1.73 ppm) and No. 2 fuel oil (LC50 = 0.651 ppm) for pink salmon fry.

In chronic 21-d exposures measured jet fuel concentrations were quite low despite constant manufacture and input of WSF due to volatilization and biodegradation (see Part III, Section III.A). The highest concentration attained was 0.42% MaxWSF (range 0.22-1.21). All fish survived at the tested concentrations with no significant differences in either weight ($p = 0.79$) or SL ($p = 0.92$) at the end of the test. No behavior differences between exposure groups were noted.

Results of the acute toxicity study for the WSF SD JP-4 were very similiar to those for P JP-4, with a calculated 96-h LC50 of 23.3% (95% FL = 21.7 to 24.7%) MaxWSF. Again, the toxicity curve was steep with a slope of 17.14±3.42.

Juvenile fish exposed to WSF SD JP-4 for 21 d were not significantly different from controls in SL ($p = 0.73$) or weight ($p = 0.54$). However, in the highest concentration attained, 0.95% MaxWSF (Range 0.06-9.78), a gross behavioral change was observed. After 10 d, fish in the 0.95% group developed a tremor and did not feed. All fish in this group were affected. These fish gradually improved over 4 d. On day 15, they no longer had a tremor and resumed feeding. Benzene and toluene are known to act as neurotoxins to fish (Buikema and Hendricks 1980). These components may have contributed to the development of the tremor. The apparent recovery of the fish could be attributed to the induction of enzymes assisting in the metabolism of active components.

C. Ventilatory Study

There was a significant linear relationship between ventilatory rates and concentration of WSF P JP-4 ($p<0.01$, $r^2 = 0.72$, Figure 10). A comparison of the change in ventilatory rate compared to change in concentration showed the same general trend ($p<0.01$, $r^2 = 0.71$, Figure 11).

A preliminary statistical analysis compared ventilatory rates for groups of exposed fish to those for control fish as blocks using ANOVA procedures. This is similar to procedures used by other investigators (e.g., Bloem 1982). Results of this statistical analysis are shown in Table 1.

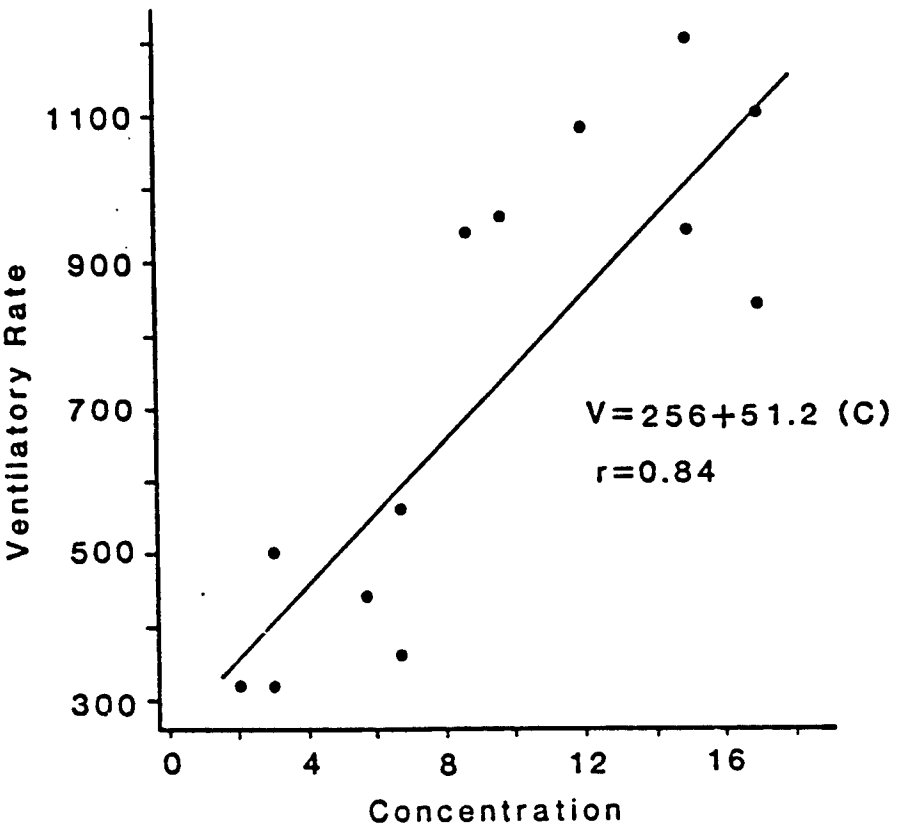


Figure 10: Ventilatory rate as a function of WSF JP-4 concentration.

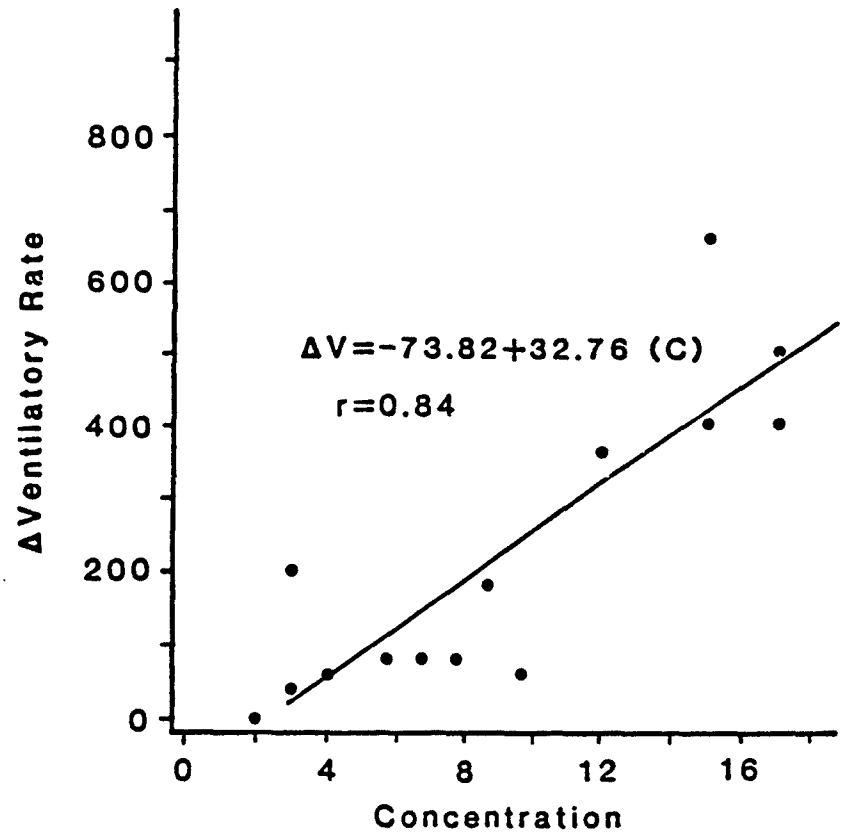


Figure 11: Ventilatory rate change as a function of WSF JP-4 concentration.

TABLE 1

Probability values (ANOVA) for ventilatory rate change in bluegill exposed to WSF P JP-4. Concentrations are expressed as % MaxWSF

Concentration	n	p-values Exposed	Control
8.5	8	0.67	0.14
10.0	5	0.10	0.50
12.0	4	0.10	0.99
14.0	5	0.14	0.95
15.0	8	0.01	0.60
17.0	3	0.02	0.70

*Significance level = $p<0.05$.

The threshold for a shift in ventilatory rate was determined to be ca. 15% WSF P JP-4 based on this analysis. However, individual variability masked the differences that were seen in the same fish between control and exposure periods.

A second statistical analysis treated fish individually by comparing median ventilatory rate for the last 4 h of control data to the first 6 h of exposure data for each fish after deleting 2 h of exposure data to allow time for WSF P JP-4 to replace the contents of each exposure chamber completely. A statistically significant diurnal variation in ventilatory rates of control fish was noted, and a different strategy was used to analyze data. Comparisons were made on 4 h of data for exposed fish and control data collected for each individual fish during the same 4-h period of the previous day (Figure 12).

These data were not always normally distributed in contrast to those of Nunn et al. (1982). Data for some fish had the null hypothesis of normality rejected at a $p = 0.05$ level of confidence. While the skewness ($p = 1.022$) and kurtosis ($p = 0.395$) of the distribution showed little appreciable deviation from normality, a conservative nonparametric paired rank sum procedure was used to compare differences in median ventilatory rate before and after exposure (Hollander and Wolfe 1973). This analysis was performed on all data.

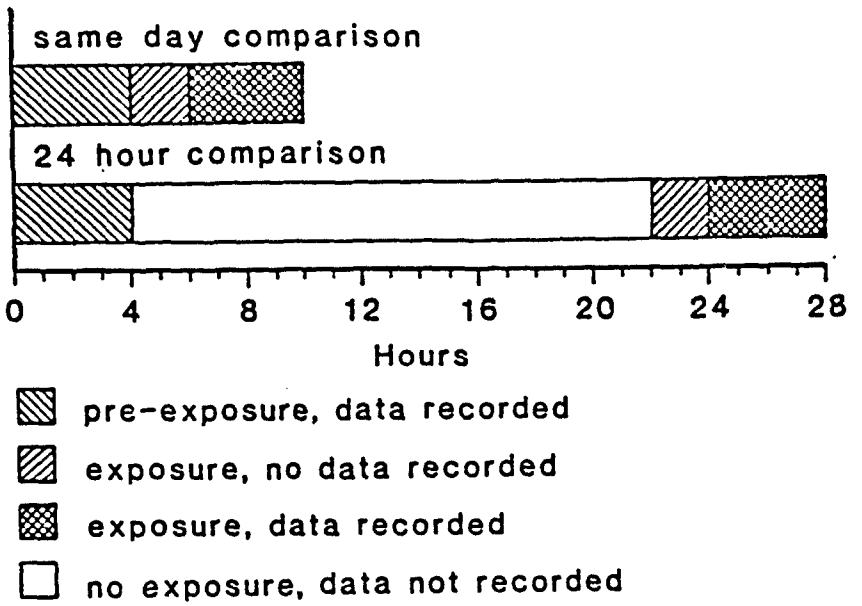


Figure 12: Display of comparative time periods used to analyze ventilatory data.

Results of this statistical analysis on ventilatory data are given in Table 2. The 24-h studies were more valid.

Using a p = 0.05 level of confidence as a cutoff, the 5.1% WSF P JP-4 concentration was determined to be the level at which a "threshold" shift in ventilatory rate could be detected. This was ca. 20% of the 96-h LC50 and less then the 96-h LC01. If same day data were considered, a similar trend was seen, however, there was more "noise" in this treatment. Changes in amplitude of ventilation responded similarly with a significant shift occurring at 5.1% WSF P JP-4 (Table 2).

A second plateau or threshold of sensitivity in ventilatory behavior seemed to occur at 15% and 17% WSF P JP-4. The actual p values were an order of magnitude lower than those for 5.1% to 12% WSF P JP-4. Control fish at these higher concentrations also had significantly elevated ventilatory rates. There was no obvious reason for this shift in the ventilatory rate in the control fish, i.e., a change in experimental procedure or other external causes. It is hypothesized that this was a "sympathy" response to the more agitated behavior of the exposed fish. As the chambers were partially transparent, fish could observe changes in behavior of other fish. The concentration of this secondary threshold correlated well with the ANOVA test discussed previously (Table 2) that demonstrated a significant effect

TABLE 2

Probability values for ventilatory rate comparisons of bluegill exposed to WSF P JP-4. Concentrations are expressed as % MaxWSF

Concentration	Number of fish	Exposed		Control	
		Vent.	Amp.	Vent.	Amp.
2.1	9	0.36	0.22	-	-
2.8	7	0.15	0.22	0.25	0.25
3.1	7	0.11	0.29	0.11	0.34
5.1	7	<0.05	0.02	-	-
6.7	9	<0.01	0.06	0.23	0.50
8.5	8	<0.01	<0.01	0.23	0.16
12.0	4	0.06	0.19	0.50	0.34
15.0	13	<0.01	<0.01	0.02	0.29
17.0	8	<0.01	<0.01	<0.01	0.45

[a]Significance level = $p < 0.05$.

when the fish were analyzed as groups. It is possible that there was a change in effect on the fish, i.e., that a different physiological effect was taking place at the higher WSF P JP-4 concentration.

Earlier ventilatory studies of Thomas and Rice (1979) also showed an increase in ventilatory rate when salmon were exposed to petroleum hydrocarbons, but those investigators used much higher relative concentrations, ca. 80% of LC50s, of Cook Inlet Crude and No. 2 fuel oils. Brocksen and Bailey (1973) reported increased ventilatory rates for salmon and striped bass exposed to benzene. It is possible that increased ventilatory rate in this study was the result of an increased metabolic rate that could be partially due to the stress and also to the actual metabolic detoxification of the ingested and absorbed petroleum components.

Results for the WSF SD JP-4 were even more pronounced in the reaction of the fish to low concentrations. As shown in Table 3, there was a significant shift in ventilatory rate from 3.6% WSF SD JP-4 to the highest level tested of 12% WSF SD JP-4. Apparently the fish were more sensitive to the shale derived fuel, or some fraction of it. There was no significant shift in the amplitude until 8.4% WSF SD JP-4 was reached. There were no significant shifts in ventilatory rate in control fish in any of these studies.

TABLE 3

Probability values for ventilatory rate comparisons of bluegill exposed to SD P JP-4. Concentrations are expressed as % MaxWSF

Concentration	Number of fish	Exposed		Control	
		Vent.	Amp.	Vent.	Amp.
1.5	9	0.06	0.19	0.50	0.28
3.6	9	0.05	0.50	0.41	0.22
8.4	9	0.04	0.42	0.41	0.03
9.7	9	<0.01	0.05	0.11	0.41
12	9	<0.01	0.02	0.23	0.47

*Significance level = $p<0.05$.

D. Preference-Avoidance Studies

There were two >5-h replicates in which fish were not exposed to any WSF P JP-4; these were controls for fish behavior over time. No significant difference occurred in the side of the exposure chamber that fish chose over time ($p = 0.62$ for "exposure side"). There was also no significant change in the number of movements over time in these control runs ($p = 0.29$, Figure 13).

In the first study, fish were exposed to dilutions of up to 12% WSF P JP-4. In this study fish spent significantly different amounts of time on the exposed side of the chamber ($p<0.01$, Figure 14). There was no difference in movement of fish at different concentrations ($p = 0.21$). When avoidance reactions at different exposures were analyzed with a LSD procedure it was determined that significantly less time ($p<0.05$) was spent on the JP-4 influenced side when fish were exposed to 3.5% and 4.9% WSF P JP-4 as compared to controls (0% WSF P JP-4), lower (0.4%, 1.3% and 2.2% WSF P JP-4) and higher (12.2% WSF P JP-4) concentrations. Fish showed no difference in behavior at the highest concentration when compared to controls. There are at least two possible explanations for this observation. First, fish olfactory organs were rapidly desensitized physiologically to WSF P JP-4. Such a hypothesis has been advanced by Norton et al. (1982) and Gardner (1978). Second, fish

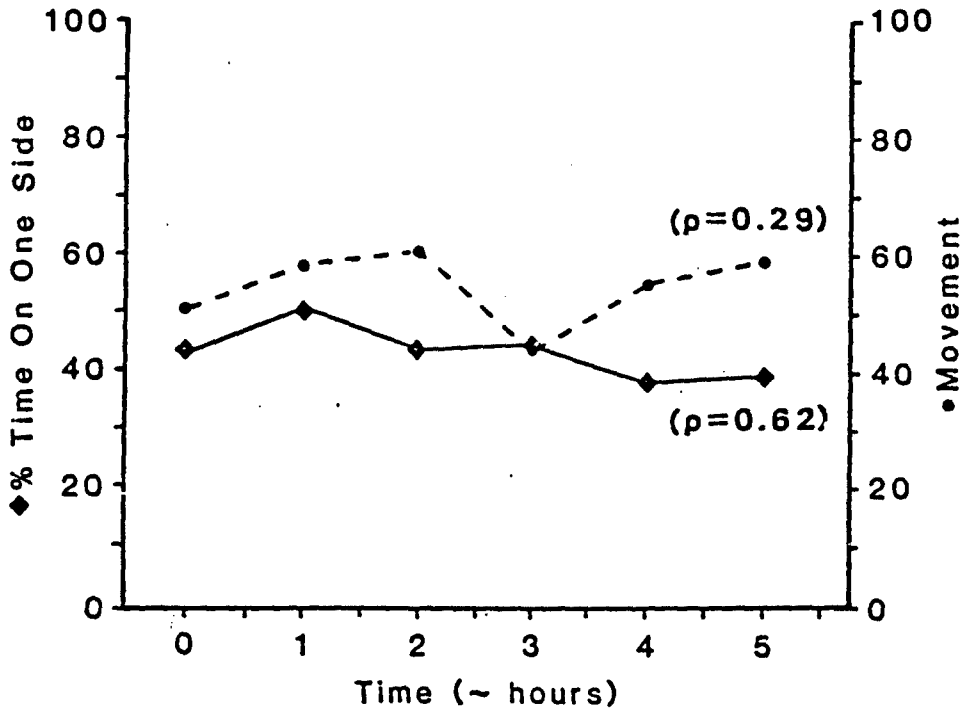


Figure 13: Control data for bluegill preference-avoidance studies.

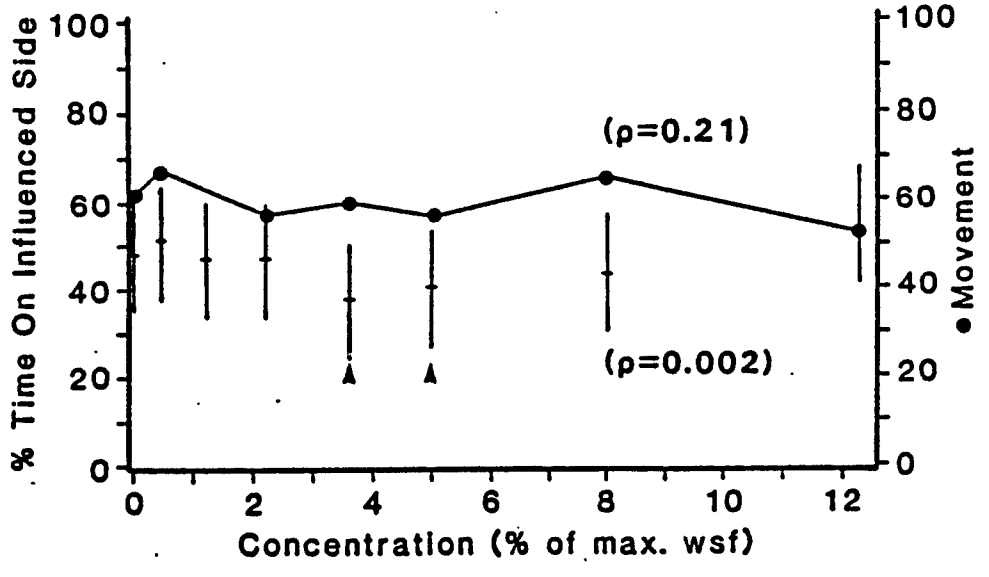


Figure 14: Effects of WSF JP-4 on preference-avoidance behavior of bluegill: Study 1.

became acclimated to WSF P JP-4 as the level slowly increased in the preference-avoidance exposures. In order to test for this possibility, the next preference-avoidance study was conducted.

In the second study, fish were exposed to the control, 0% WSF P JP-4, and then to the highest level of exposure from Study 1, 12% WSF P JP-4. If acclimation affected fish behavior in Study 1, it was hypothesized that the fish would actively avoid sudden exposure to a high concentration of WSF P JP-4. Fish showed no significant difference in avoidance or movement behavior when exposed to 12% WSF P JP-4 (Figure 15).

The third study was conducted with more rapidly increasing and higher concentrations of WSF P JP-4 than Study 1 in order to determine fish behavior when exposure level approached the 96-h LC50. There was no significant difference in either avoidance behavior or movement ($p = 0.08$ and 0.06, respectively; Figure 16). This may partially be due to the rapidity of increasing concentration and high level of "noise" in fish behavior due to a fewer number of observations. It should be noted that these p values were close to a 0.05 significance level, and avoidance behavior at median levels of concentration, 3.7% and 5.2% WSF P JP-4, was distinctly greater than for control and high (23.3% WSF)

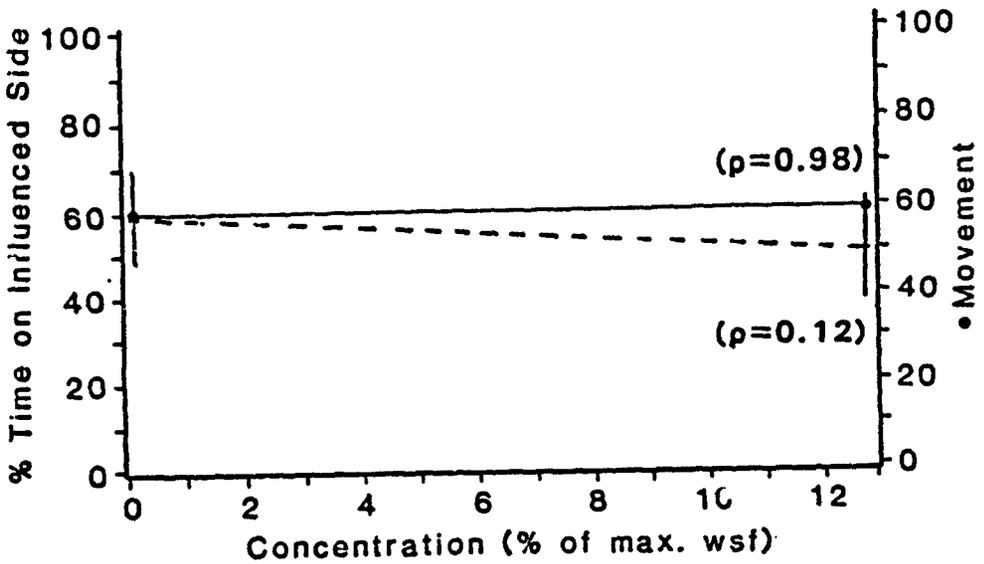


Figure 15: Effects of WSF JP-4 on preference-avoidance behavior of bluegill: Study 2.

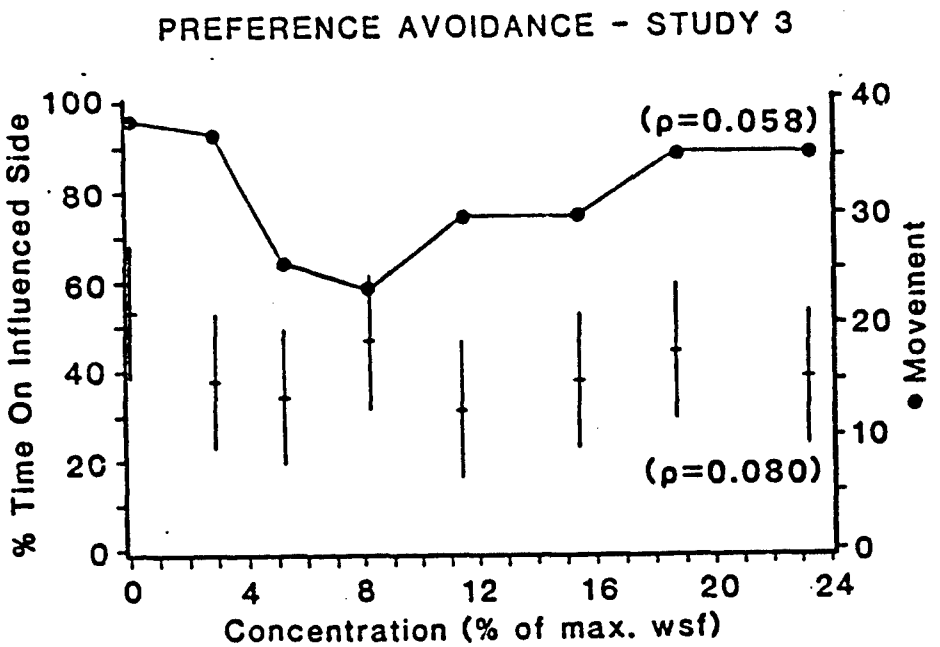
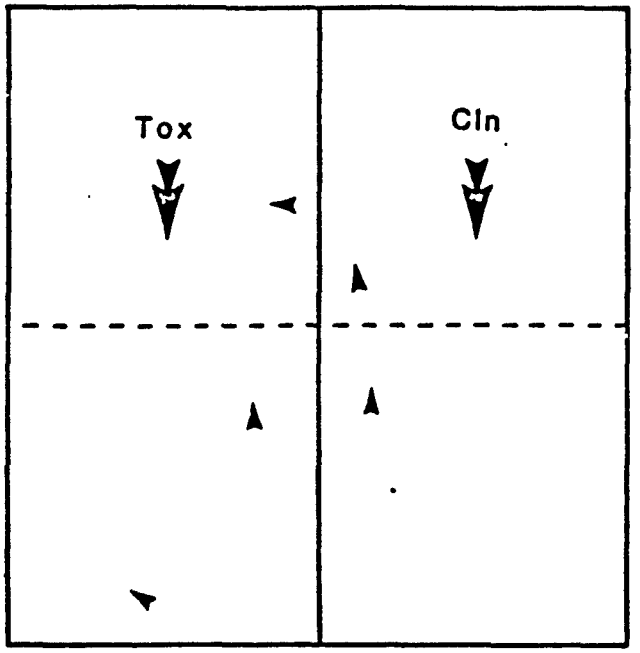


Figure 16: Effects of WSF JP-4 on preference-avoidance behavior of bluegill: Study 3.

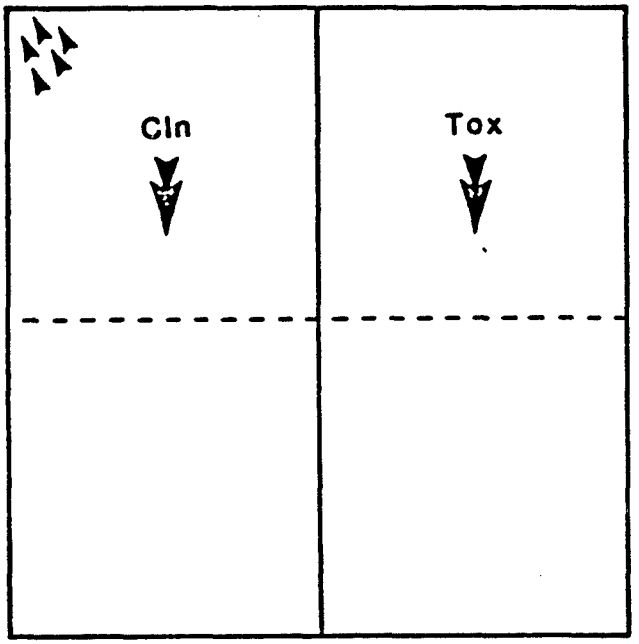
concentrations. Study 3 results confirmed those of Study 1, although the level of significance was different.

Although difficult to quantify, there was an apparent difference in general behavior of fish during these studies. When fish were first placed in the chamber, they seemed to swim randomly with little or no schooling behavior. When WSF P JP-4 reached the median levels at which the avoidance behavior was manifested, attempts by fish to escape the preference-avoidance arena entirely were more noticeable. The more normal, random swimming pattern is shown on the left and the "escape behavior" is shown on the right in Figure 17. This behavior was discernable with most groups of exposed fish.

Juvenile salmon have been shown to avoid 1.9 to 3.7 mg/l total petroleum hydrocarbons (Maynard and Weber 1981). Adult salmon have been shown to avoid moderate to high concentrations of petroleum hydrocarbons in a fish ladder choice study in the natural environment (Weber et al. 1981). Other workers have reported fish that did not avoid petroleum contamination in a marine environment that was so severe that the water was cloudy with suspended petroleum. In related studies, Folmar et al. (1981) noted decreased predation by coho salmon; this could be attributed to failure of olfaction. Many species of fish have been



A: Normal behavior of fish in preference/avoidance chamber.



B: "Escape" behavior of fish in preference/avoidance chamber.

Figure 17: "Escape behavior" of bluegill at moderate levels of WSF JP-4.

shown to depend heavily on olfaction for feeding (Kleerekoper 1969).

Results of the preference-avoidance studies with the SD JP-4 were not very pronounced. There were no significant shifts in either the choice of side during the exposures or in the number of movements as seen in Figure 18. This may be partly due to the shorter number of observation periods or to a more rapid desensitization or neural disturbance of the olfactory apparatus.

E. Blood Chemistry/Physiology

A data base for "normal" physiology was collected before fish were exposed to WSF P JP-4. These data are presented in Table 4. Most of these parameters were not normally distributed. This problem was discussed by Miller et al. (1983). Because of non-normality, data were reanalyzed with a nonparametric procedure (Brunden et al. 1970). The same Wilcoxon rank sum nonparametric procedure used for analysis of the ventilatory data was used. In Table 5, median values and 95% confidence limits are presented for the different physiological parameters.

Fish were initially exposed to 13% WSF P JP-4, half the nominal 96-h LC50. This concentration was equal to the 96-h LC04 based on the log-probit curve but was originally chosen

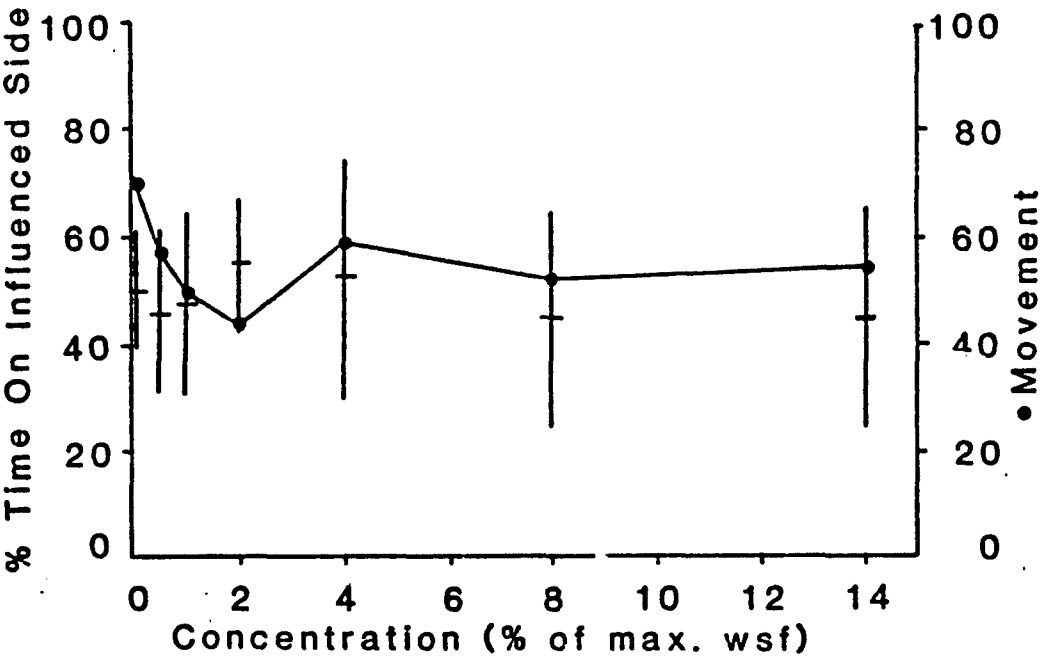


Figure 18: Effects of WSF SD JP-4 on preference-avoidance behavior of bluegill.

TABLE 4

Physiological parameters of control bluegill

Parameter	n	Mean	s	CV	s^2	Prob:0	99%	1%	Range
Size									
Length	80	4.77	0.59	12.4	0.35	<0.01	6.80	2.70	4.10
Weight	79	58.0	28.0	49.0	800.	<0.01	179.0	31.5	151.0
Mucous									
Ketosis	33	0	-	-	-	-	-	-	-
Hemolysis	33	5.85	2.73	46.7	7.40	<0.01	9	0	9
Muscle									
Water content-flesh (%)	33	78.9	0.007	0.92	<0.0001	<0.01	75.7	72.7	3.0
Blood									
Hemoglobin (g/100ml)	72	7.0	1.1	16.3	1.3	0.13	9.5	4.4	5.1
Hematocrit (%)	80	38.6	6.6	17.1	43.7	>0.15	53.9	25.0	28.9
RBC ($x10^6/mm^3$)	58	1.59	31	20	1000	>0.15	239	98	151
Calcium (mg/dl)	67	23.4	14.3	61	204	<0.01	99	8.5	90.5
Chloride (mg/dl)	68	138	88	63	7746	<0.01	516	33	482
Magnesium (mg/dl)	61	4.3	1.9	44.1	3.6	<0.01	13.9	2.4	11.5
Phosphorous (mg/dl)	69	28.7	13.2	45.9	173.5	<0.01	81.0	13.8	67.2
Total protein (g/dl)	78	5.0	1.6	31.7	2.6	<0.01	9.4	2.7	6.7
Albumin (g/dl)	75	1.77	0.54	30.27	0.29	<0.01	4.4	0.85	3.55
LDH (mg/l)	73	2.884	0.15	0.54	2400	0.06	8.106	0.323	7.783
SGOT (ug/l)	60	.524	166	0.32	27.78	0.02	259	0.259	0.774
Glucose (mg/dl)	24	71.9	19.0	26.4	360	<0.01	128	43.6	84.4
Liver									
Water content (%)	33	77.0	0.04	5.02	0.002	0.28	71.8	82.9	11.1
ATP (umoles/ml)	30	3.22	0.42	20.6	0.38	0.03	4.81	2.12	2.69

TABLE 5

Nonparametric analysis of control fish blood parameters

Parameter	n	Median	95% Confidence Interval Lower Limit	Higher Limit
Size				
Length	33	8.0	4.5	8.0
Mucous				
Ketosis	33	0	-	-
Hemolysis	33	7.00	6.33	8.00
Muscle				
Water content-flesh (%)	33	75.2	74.7	75.4
Blood				
Hemoglobin (g/100ml)	72	7.1	6.9	7.4
Hematocrit (%)	80	38.0	36.5	40.5
RBC ($\times 10^6/mm^3$)	58	1.49	1.60	1.66
Calcium (mg/dl)	67	18.4	14.7	25.2
Chloride (mg/dl)	68	102.2	98.5	110.0
Magnesium (mg/dl)	61	4.0	3.8	4.3
Phosphorous (mg/dl)	69	25.2	21.7	30.0
Total protein (g/dl)	78	4.60	4.30	5.1
Albumin (g/dl)	75	1.60	1.50	1.80
LDH (mg/l)	73	2.553	2.016	3.390
SGOT (ug/l)	60	.506	0.416	0.577
Glucose (mg/dl)	24	69.0	62.8	73.6
Liver				
Water content (%)	33	70.0	69.6	71.0
ATP (umoles/ml)	18	3.22	2.60	3.78

on the basis of the initial ANOVA block analysis discussed previously that showed that this concentration was the threshold for a shift in ventilatory rate (Table 1). As this exposure was for only 96 h it was not likely that many physiological changes would be seen. A second study was conducted with an exposure concentration that approximated the 96-h LC50 of 26% WSF P JP-4. This study should detect changes that could be expected in fish just before the concentration became lethal. This second exposure lasted for 24 h due to the anticipated high mortality of exposed fish.

At the higher concentration, the expected kill of ca. 50% of the adult fish occurred. This confirmed that previous lethality determinations, based on experiments with juvenile fish, were applicable to adult bluegills. Although no narcosis of fish was seen at the sublethal concentrations used for preference-avoidance studies, there did seem to be such an effect in this study. Just before fish expired, they were suspended head up/tail down in the water column. This behavior was also seen in the acute toxicity tests before death of test fish.

Control fish were subjected to the same procedures previously described except they were not exposed to WSF P JP-4 during observation. When the two sets of control fish were compared, there were significant differences between

them (Table 6). No extrinsic reasons for these differences were identified. Fish were collected at the same time from the same source, had been similarly treated, and showed no signs of disease. Tests were run in close time proximity so no effects due to seasonality were suspected. Possibly the differences were due to the different lengths of holding time under test conditions.

Because the control fish were significantly different from each other, the exposed fish for each study were compared to their own respective group of controls (Table 7). A comparison between the exposed fish for each study and the data base for all nonexposed fish yielded the same final results that are discussed next, so the differences between the sets of control fish did not affect the final outcome of the entire study, it only confounded the interpretation of the significance of physiological data. The physiological results for these two exposure studies are given in Table 7. Measured values are presented as median values, with p values generated by the nonparametric Wilcoxon rank sum procedure.

The 13% WSF P JP-4 resulted in no significant differences between control and exposed fish for the "whole body" parameters: hemolysis, ketosis, and percent water content of muscle (Table 7). At the higher concentration, 26% WSF P JP-4, there was a significantly greater percent water

TABLE 6

Comparison of control fish data for two exposure studies

Parameter	Control 13% WSF JP-4	Control 26% WSF JP-4	p-value
Size			
Length	4.4	4.4	0.42
Weight	45.7	41.8	0.92
Mucous			
Ketosis	0	0	-
Hemolysis	5.0	6.8	0.05
Muscle			
Water content-flesh (%)	75.0	76.0	0.82
Blood			
Hemoglobin (g/100ml)	8.1	7.0	<0.01
Hematocrit (%)	46.3	40.6	<0.01
RBC ($x10^6/mm^3$)	1.37	1.54	0.18
Calcium (mg/dl)	38.4	30.0	<0.01
Chloride (mg/dl)	101.6	93.6	0.26
Magnesium (mg/dl)	4.2	4.1	0.40
Phosphorous (mg/dl)	30.0	24.6	0.06
Total protein (g/dl)	7.0	6.2	0.01
Albumin (g/dl)	2.4	2.0	<0.01
LDH (mg/l)	5.000	3.826	0.04
SGOT (mg/l)	0.483	0.510	0.96
Glucose (mg/dl)	66.8	70.5	0.22
Liver			
Water content (%)	75.0	70.0	<0.01
ATP (umoles/ml)	3.86	2.57	0.02

TABLE 7

Physiological and blood parameters in bluegill exposed to WSF JP-4

Parameter	13% WSF JP-4			26% WSF JP-4		
	Control	Exposed	p-value	Control	Exposed	p-value
Mucous						
Ketosis	0	0.3	0.39	0	0	-
Hemolysis	5.0	5.5	0.76	6.8	6.5	0.41
Muscle						
Water content (%)	75.3	75.3	0.14	76.1	77.3	0.01
Blood						
Hemoglobin (g/100ml)	8.1	8.2	0.71	7.0	4.0	0.01
Hematocrit (%)	46.3	44.4	0.67	40.6	33.8	0.04
Red blood cells ($x10^6/mm^3$)	1.37	1.70	0.23	1.48	0.98	<0.01
Calcium (mg/dl)	38.4	41.3	0.82	30.0	28.0	0.44
Chloride (mg/dl)	101.6	83.4	0.05	93.6	78.4	<0.01
Magnesium (mg/dl)	4.5	5.0	0.50	4.1	4.0	0.75
Phosphorous (mg/dl)	30.0	34.2	0.17	24.6	29.4	0.04
Total protein (g/dl)	7.0	7.0	0.08	6.2	5.8	0.20
Albumin (g/dl)	2.4	2.6	0.21	2.0	1.8	0.22
Lactate dehydrogenase (mg/l)	5.000	4.914	0.71	3.826	5.153	0.21
Aspartate aminotransferase (ug/l)	0.482	0.601	0.17	0.510	1.053	<0.01
Glucose (mg/dl)	66.8	91.4	0.01	70.5	439.	<0.01
Liver						
Water content (%)	69.8	71.0	0.05	70.0	77.5	<0.01
ATP (nmoles/ml)	3.86	2.90	0.01	3.86	1.31	0.01

content in exposed fish muscle and no change in hemolysis or ketosis. This would suggest that fish exposed to near lethal concentrations have osmoregulatory problems.

For the three whole blood component measurements, hemoglobin, hematocrit and RBC counts, there were no significant differences observed in fish exposed to the lower WSF P JP-4 concentration. At the higher concentration, significant decreases occurred in all three parameters. The most likely explanation for this difference is a blood dilution effect due to the osmoregulatory problem mentioned previously.

A significant decrease occurred in chloride ion concentration but not in the other three serum ions measured in fish exposed to the lower WSF P JP-4 concentration. At the higher WSF P JP-4 concentration blood chloride ion concentrations were significantly lower; calcium and magnesium were not significantly changed; and phosphorous was significantly elevated. Decrease in chloride ion concentration for both exposure concentrations would again indicate an osmoregulatory problem.

There were no significant shifts in serum enzymes LDH and SGOT for fish exposed to the lower WSF P JP-4 concentration. In addition, no changes occurred in total protein or albumin levels. (Glucose was the only serum parameter that was

significantly elevated ($p = 0.01$)). At the higher WSF P JP-4 concentration there was no shift in the level of serum LDH, but the levels of both glucose and SGOT were significantly elevated. These were changes that could not be attributed to a suspected osmoregulatory problem in exposed fish. It is hypothesized that there was metabolism of accumulated JP-4 components in liver. There were sequestered materials that stained positive for lipids visible in the microscopic examination of liver. Other authors have noted the storage of conjugated and nonconjugated metabolic byproducts of toxicants (Roubal et al. 1977, 1978). This metabolism could require the increased levels of transaminase (SGOT) that were seen. More likely, the elevated SGOT levels could be the result of liver damage from exposure to WSF P JP-4. This is the clinical significance of increased SGOT in humans (Ravel 1969). Elevated LDH might also be expected in liver damage, but such elevations are reported to be much less sensitive indicators and slower to respond (Zilva and Pannall 1971).

Although no differences occurred in levels of total protein or albumin, there was a pronounced increase in the level of glucose in blood of all exposed fish. This may be the result of metabolism as mentioned previously or simply due to fish mobilizing their stores of glycogen. There did appear to be fewer glycogen deposits present in the liver of fish after exposure to WSF P JP-4. This was particularly

noticable after the longer exposure periods used for the 13% WSF P JP-4. This loss of glycogen and elevated glucose have been reported by Heitz et al. (1974) in livers of mullet exposed to Empire Mix Crude Oil.

There were significant differences at both concentrations for effects on liver. Both exposure levels resulted in increased water content of liver. This could be the result of osmoregulatory problems and perhaps of changes in liver tissue because of sequestering of metabolites. The amount of ATP present in the liver of fish exposed to both concentrations decreased significantly. These lower ATP levels may be a reflection of the higher water content of the liver. Similar results were found by Heath (1984) when fish were exposed to sublethal levels of copper.

Overall, WSF P JP-4 appears to affect osmoregulation and liver function. These effects were much more pronounced in fish exposed to the higher, 26%, WSF P JP-4 concentration. Similar results were reported by Heitz et al. (1974) who studied liver enzymes in salmon exposed to Empire Mix Crude Oil for 4 d. They also found increased levels of liver glucose that were attributed to carbohydrate metabolism. The depletion of glycogen stores was also reported by Hawkes (1979) after rainbow trout were exposed to Prudhoe Bay Crude Oil. Similar osmotic imbalances were measured as changes in chloride levels after hydrocarbon exposure and were

attributed to an alteration in cell membrane permeability in the gills (Morrow et al. 1975).

For the "whole body" parameters measured in fish exposed to WSF SD JP-4, there were significant decreases in water content in the muscle for both time periods and significantly increased ketones measured in the mucous (Table 8). The increased reading for ketosis would indicate that fish were metabolizing fatty acid stores. These changes in ketosis and the change in metabolism indicated were not seen with the exposures to WSF P JP-4. Decrease in water content of the muscle was in opposition to what was seen in the WSF P JP-4 high concentration exposure where the water content increased indicating a failure of the fish to maintain osmoregulation.

Results for the three whole blood component measurements were mixed. After the shorter exposure period, hemoglobin levels increased significantly. But, hemoglobin levels were significantly decreased after the longer exposure period. The hematocrit percentage was not significantly changed at the lower concentration, but was elevated after the longer exposure period. The RBC count was significantly reduced for the shorter period but unchanged for the longer exposure period. Mixed results like these make it difficult to state what was occurring. No difference was observed in RBCs when examined microscopically. Lowered water content of flesh

TABLE 8

Physiological and blood parameters in bluegill exposed to WSF SD JP-4

Parameter	Short SD&WSF JP-4 Control	Short SD&WSF JP-4 Exposed	Short SD&WSF JP-4 p-value	Long SD&WSF JP-4 Exposed	Long SD&WSF JP-4 p-value
Mucous					
Ketosis	0	0.24	0.02	1.04	<0.01
Hemolysis	7.0	7.0	0.62	7.0	0.57
Muscle					
Water content (%)	75.2	74.4	<0.01	73.2	<0.01
Blood					
Hemoglobin (g/100 ml)	7.1	8.1	0.03	6.03	0.09
Hematocrit (%)	38.0	41.0	0.10	43.4	<0.01
Red blood cells ($x10^6/mm^3$)	1.49	0.87	<0.01	1.64	0.95
Calcium (mg/dl)	18.4	14.2	0.02	11.0	<0.01
Chloride (mg/dl)	10.22	100.5	0.41	99.0	0.06
Magnesium (mg/dl)	4.0	3.0	<0.01	3.8	0.41
Phosphorous (mg/dl)	25.2	21.0	0.01	24.0	0.88
Total protein (g/dl)	4.6	4.1	0.12	5.5	0.15
Albumin (g/dl)	1.6	1.55	0.687	2.0	0.07
Lactate dehydrogenase (mg/l)	2.553	3.458	0.32	9.162	<0.01
Aspartate aminotransferase (ug/l)	0.506	0.720	<0.001	1.086	<0.01
Glucose (mg/dl)	69.0	81.6	0.24	71.2	0.73
Liver					
Water content (%)	70.0	76.4	<0.01	76.8	<0.01
Adenosine triphosphate	1.34	1.30	0.90	1.48	0.99

would make an osmoregulatory failure and resultant dilution effect on the blood as observed after exposure to WSF P JP-4 seem unlikely. The increased hematocrit percentage would also make the dilution effect seem unlikely.

Both exposure periods showed significant decreases in calcium, but decreases in magnesium and phosphorous occurred only in the short period. Again, as discussed previously, these different results would seem to rule out dilution effects from osmoregularity failure, at least as the only cause. Levels of SGOT were significantly increased in fish exposed for both exposure periods, but LDH only increased in fish exposed for the longer period. As mentioned previously, such increased levels of these enzymes would be considered indicative of liver damage if observed in human blood analysis. Glucose levels were not significantly shifted after either exposure period.

Water content of the liver was significantly elevated after both exposure periods; such an elevation also occurred in the studies with WSF P JP-4. No significant shifts occurred in the levels of liver ATP.

Overall, a clear effect was observed on the liver of fish exposed to WSF SD JP-4. This was demonstrated by the increased water content and in the changes in the liver produced enzymes, LDH and SGOT. Changes in the levels of

glucose in blood that were observed after exposure to WSF P JP-4 did not occur with WSF SD JP-4. Changes did occur in metabolism of fish as demonstrated by the increase in ketosis. If the increase in ketosis were due solely to starvation, such an increase would also have been observed in control fish. No other clear changes were seen in the fish after exposure to the WSF SD JP-4.

F. Histology of Fish

The lower WSF P JP-4 concentration (13%) produced no effects upon gill tissue that were discernible when the tissues were examined by light or electron microscopy. At the higher WSF P JP-4 concentration the only observed effect was an extended paranuclear space in many cells (Figure 19). Other investigators have reported hyperplasia (DiMichele and Taylor 1978; Gardner 1978) as well as cellular lesions and edema (Norton et al. 1982) in the gills of fish exposed to petroleum hydrocarbons. Lack of these effects in gill tissue after exposure to WSF P JP-4 can be partially explained by the fact that at this higher concentration the exposure period was only 24 h. This might not have been a sufficient time for notable tissue changes. Solangi (1980) stated that many histological changes were not manifested until after several days (10-15) of exposure. This would seem to be especially true in looking for such effects as hyperplasia where new cellular material must be generated.

Figure 19: Electron micrographs of gill tissue from control and WSF P JP-4 exposed bluegill. N = nucleus; PNS = paranuclear space; RBC = red blood cell. Micrograph of control is on left.

Preliminary investigations with longer, near lethal exposures had resulted in an increase in ruthenium red staining mucopolysaccharide materials in the gill (Doane, unpublished data). Similar results were reported by Norton et al. (1982). Mucous cells in the gills of fish exposed to the high concentration in this study appeared to be normal with no abnormal buildup of mucous (Figure 20).

The livers of fish exposed to both concentrations of WSF P JP-4 were paler and of different texture than those from control fish. At 100x light magnification, many small lipid staining vacuoles appeared in the hepatocytes of livers of fish exposed to both concentrations. These were assumed to contain sequestered metabolites of P JP-4. Few similar vacuoles appeared in the hepatocytes of livers from control fish. Reports have recorded of such stored metabolic products after exposure to petroleum hydrocarbons (Roubal et al. 1977, 1978; Solangi 1980). There was also a discernible reduction in amounts of stored glycogen in the livers of fish exposed to the lower concentration but not in those exposed to the higher concentrations of WSF P JP-4. As mentioned previously, this correlated well with increases in glucose levels. Similar findings were reported by Hawkes (1979) and Solangi (1980). These lipid vacuoles were also visible in the electron micrographs of exposed liver cells (Figure 21). A greater separation also appeared between individual liver cells in the exposed fish. These spaces

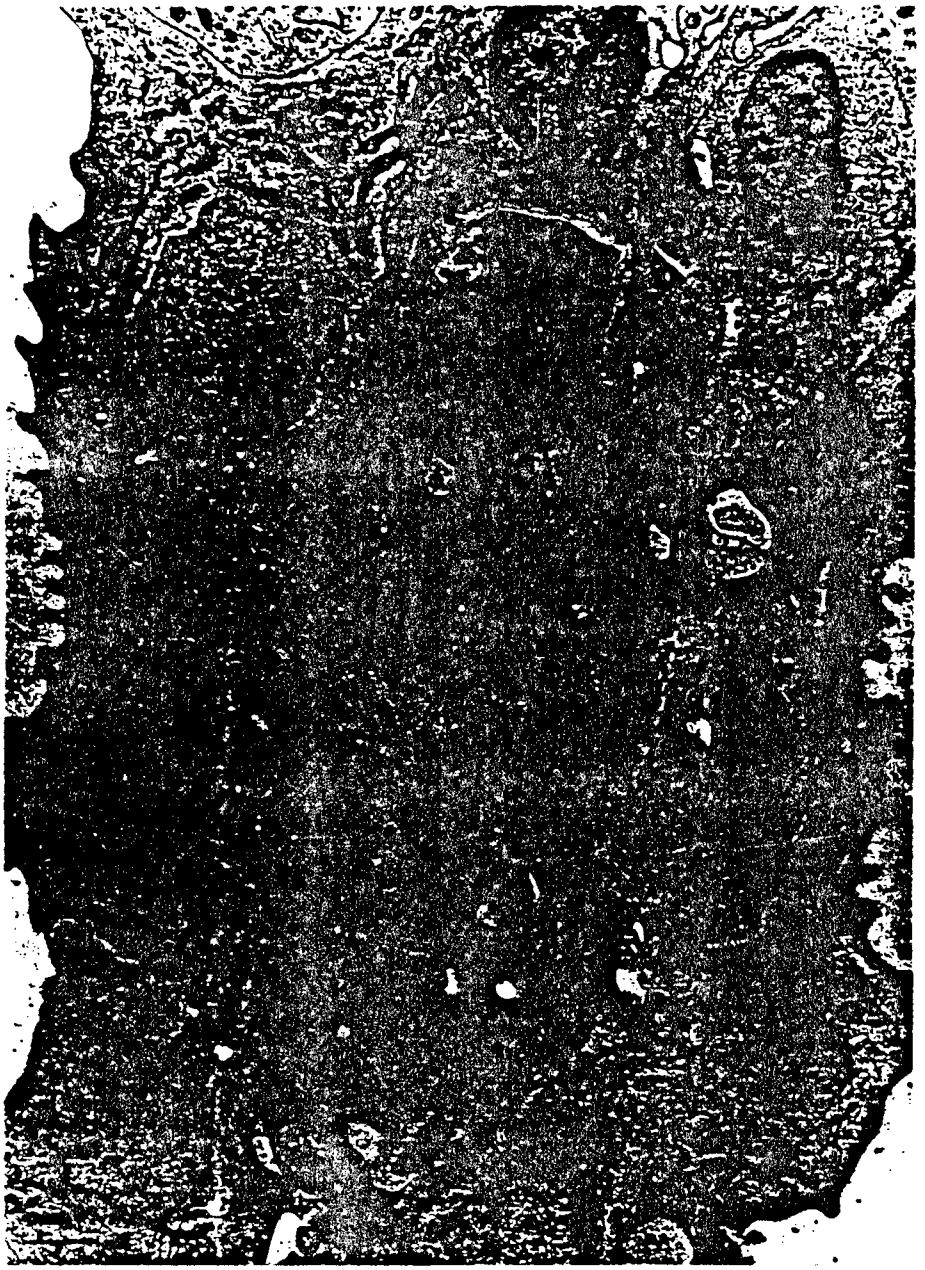


Figure 20: Electron micrograph of mucous cell from gill of fish exposed to 26% WSF P JP-4. MC = mucous cell; MV = cytoplasmic mucous vacuole; N = nucleus; PNS = paranuclear space.

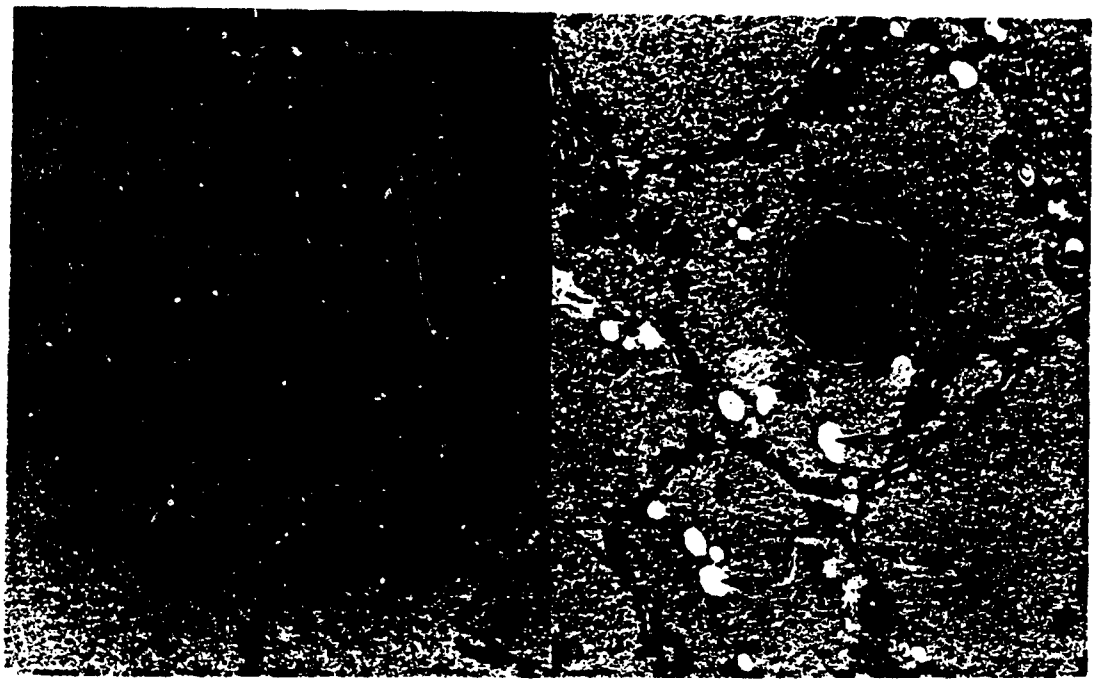


Figure 21: Electron micrograph of liver tissue from control and WSF P JP-4 exposed fish. CM = cell membrane; ER = endoplasmic reticulum; G = glycogen deposits; I = intercellular space; M = mitochondria; N = nucleus; V = vacuole. Micrograph of control is on left.

were electron dense and were suspected of also having sequestered JP-4 component materials. Although other investigators have reported changes in the general amount of endoplasmic reticulum in liver cells exposed to petroleum hydrocarbons (Hawkes 1979), this did not seem to be the case in the livers of fish exposed to WSF P JP-4. In an effort to substantiate these impressions of relative changes in glycogen levels, stored metabolites, and no changes in other cell organelles, a random planimetry method (see Part II, Section II.G) was used to collect data that could be statistically analyzed. Results of t-tests on the relative number of positive "counts" for nine electron micrographs of control and 11 of exposed fish livers for these cellular components are given in Table 9. This statistical treatment, although performed on a relatively few number of electron micrographs, supports the visual impressions discussed previously. The category of Nucleus was included as a check on the similarity of cell types between control and exposed fish. If there were no differences in "counts" for cell nuclei, as was the case, it was hypothesized that the random electron micrographs were taken of similar cells in the respective livers. Also, although this was not apparent in these livers, Solangi (1980) had reported swollen nuclei in livers of fish exposed to Empire Mix Crude Oil WSF.

TABLE 9

Results of t-tests on quantities of cellular components of control and 26% WSF JP-4 exposed fish liver

Components	"Counts"[1] Control	Exposed	p-values
Vacuoles	0.33	2.36	<0.01
Endoplasmic reticulum	12.45	15.45	0.18
Mitochondria	3.11	3.27	0.87
Nucleus	3.22	2.37	0.24
Glycogen	28.89	28.54	0.94

[1] "Counts" = Presence of hole punched in cellular component in electron micrograph

Page 76 and 77
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2 Feb 90

However, long-term exposure to 0.95 % MaxWSF of SD JP-4 appeared to be neurotoxic.

Increased ventilation rate in exposed bluegill sunfish was detected at a sublethal concentration of 5.1% WSF P JP-4. This was equivalent to 20% of the nominal 96-h LC50 but less than the 96-h LC01. Biomonitoring of ventilatory rate of bluegill was therefore an excellent method for approximating the NOEC. There were few significant adverse physiological effects at an exposure concentration several times higher. Reaction of the fish to WSF SD JP-4 was even more pronounced-- a significant shift in ventilatory rate occurred at concentrations above 1.5% and a shift in ventilatory amplitude at 9.7% MaxWSF.

A second biomonitoring procedure, preference-avoidance behavior, proved to be a much less sensitive method of detecting sublethal levels of WSF P JP-4. There was a significant increase in avoidance behavior at concentrations similar to those that caused a change in ventilatory behavior. However, this avoidance behavior was not observed at higher concentrations. There was no indication of narcosis or anesthetization of fish during the preference-avoidance studies. This would indicate that the preference-avoidance behavior technique was not reliable for detecting the presence of jet fuel in water or detecting the NOEC. It was hypothesized that this was due to damage to

fish olfactory tissue. No significant shift occurred in avoidance or movement behavior of fish exposed to WSF SD JP-4 at any concentration studied.

There were few significant shifts in physiological and blood parameters measured on fish exposed to a WSF P JP-4 concentration that was half the nominal 96-h LC50. Several changes occurred in physiological measurements for fish exposed to a higher concentration that was essentially equal to the 96-h LC50. These changes included increases in liver and muscle water content, and decreases in serum chloride indicating osmoregulatory problems; increases in SGOT, indicating possible liver damage; and increase in serum glucose, indicating a generalized stress syndrome. These exhibited effects are those that just precede mortality. This would indicate that blood parameter analyses were not effective means for determining sublethal toxicity in bluegill exposed to WSF P JP-4 for short-term exposures. Even at the LC50, most significant changes may be attributed to osmoregulatory problems. Several changes were seen in fish exposed to the WSF SD JP-4. More definite liver damage was noted than for the WSF P JP-4. Problems with osmoregulatory balance were not as pronounced, even at the longer exposure period.

The only pronounced effect of WSF P JP-4 exposure seen in histological examination of gill and liver tissue was the

sequestering of suspected metabolites of WSF P JP-4 in lipid vacuoles in the liver. Slight effects were observed in increased paranuclear space in gill tissue and separation of cell membranes in liver tissue. No differences were observed in endoplasmic reticulum, mitochondria, or nuclei of liver tissue from control and exposed fish. Effects of WSF SD JP-4 exposure were quite similar. This indicates that the use of ultramicroscopy is not an effective method for evaluating sublethal damage to organisms exposed to WSFs of JP-4s.

These results clearly indicate that biomonitoring of bluegill exposed to WSFs of JP-4s using ventilatory rate changes is an effective method and is superior to preference-avoidance testing and physiological or histological studies.

PART III

I. INTRODUCTION

A. Contents

This is Part III of the third and final annual report for AFOSR Grant 82-0059 and deals with the acute and chronic effects of the water soluable fraction (WSF) of petroleum derived (P) and shale derived (SD) JP-4 jet fuel on aquatic invertebrates and microbial communities.

An abstract of research results is found in Part I of this report.

B. Background

Attempts were made to define acute and chronic effect levels for three aquatic invertebrates, the oligochaete, Aeolosoma headleyi, the cladoceran, Daphnia pulex, and the insect, Paratanytarsus parthenogenica. In addition, possible effects on naturally derived microbial communities were studied. When combined with toxicity determinations for fish presented earlier in this report, these data provide a broad base for evaluations of hazard or relative toxicity of the two jet fuels to aquatic life.

II. MATERIALS AND METHODS

A. Toxicity Tests With Invertebrates

Conditions for acute tests are summarized in Table 10. All tests were conducted with the Mount-Brungs dilutor (Figure 3) modified to accomodate aquatic invertebrates by reducing test volumes and minimizing turbulence of flow. An aliquot of 225 ml of each concentration of WSF was delivered to a 1-l glass beaker every 20 min. Material in these central beakers then siphoned into three replicate polyethylene test containers (102 mm diameter and 76 mm height) containing 325 ml test media. The 2-mm inside diameter of the siphon tubes slowed the flow of test material into the test chambers and minimized turbulence. Test material left the test chambers through a V-notch covered by 200 um mesh nylon screen. All components of the system were covered by sheet glass or plastic film. This system provided a replacement volume of 325 ml every 1.5 h. Light was provided by a combination of Durotest Optima and Vitalite fluorescent bulbs at an intensity of 20 ft-c at the air-water interface on a photoperiod of 16 h light and 8 h dark. Diluent water was carbon dechlorinated Blacksburg municipal tap water. Acute toxicity tests were conducted at ambient temperatures (e.g., 20-28 °C). The end point was death after a 48-h exposure. Where possible, acute toxicity test data were analyzed using probit analysis to calculate LC50s (Finney 1976). When an

TABLE 10

Conditions for chronic toxicity tests

Organism	Test Material	Test Type[1]	Temperature (°C)	Food or Substrate (mg/l)[2]	Test Length (d)	Organism Age/Stage	Response Measured
Aeolosoma	PJP-4	D, FT	23-25	200 TC	7	small	Population growth
Aeolosoma	SD JP-4	D, FT	22-25	200 TC	7	small	Population growth
Daphnia	PJP-4	D, FT	20-22	2.5 A	21	<24 h old	Survival, growth, reproduction
Daphnia	SD JP-4	D, FT	22-24	15 A	21	<24 h old	Survival, growth, reproduction
Paratanytarsus	PJP-4	D, FT	21-24	15 TC monolayer S	28	egg	Survival, growth, reproduction
Paratanytarsus	SD JP-4	P, SR	20-22	15 TC	4	egg	Hatching
Microbial Communities	PJP-4	D, FT	23-28	PF	10	barren, uncolonized	Colonization, Autotrophic Index
Microbial Communities	SD JP-4	D, FT	22-26	PF	10	barren, uncolonized	Colonization Autotrophic Index

[1] D=definitive, P=preliminary, SR=static-renewal, FT=flow-through

[2] TC=trout chow suspension; A=algae, i.e. Chlamydamonas; PF=polyurethane foam; S=builders sand

LC50 could not be calculated, the percent survival in the highest concentration obtained was reported.

Conditions for chronic tests were similar to those for acute tests and are summarized in Table 11. Chronic toxicity tests monitored survival, growth, and/or reproduction for longer periods of time; the length of time depended on the life-cycle of the test organism. Chronic effects of WSFs on the survival of test organisms were evaluated using contingency table analysis to detect significant differences from the control. Effects on growth and reproduction were evaluated using ANOVA. Differences between groups were located with Duncan's multiple range test when sample sizes were equal. Scheffe's multiple comparison technique was used for unequal sample sizes. Relationships between dose and response were examined with ordinary least squares regression analysis.

Several indices of chronic effect levels were calculated. No observable effects concentrations (NOEC) were defined as the highest concentration tested showing no statistically significant difference from the control. Similarly, lowest observable effects concentrations (LOEC) were defined as the lowest concentration tested showing such a difference. The maximum allowable toxicant concentration (MATC) was calculated as the geometric mean of the NOEC and LOEC. Where a significant relationship occurred between dose and

TABLE 11

Conditions for acute toxicity tests

Organism	Test Material	Test Type[1]	Temperature (°C)	Food or Substrate (mg/l)[2]	Test Length (d)	Organism Age/Stage	Response Measured
Aeolosoma	PJP-4	D, FT	25	450 TC	2	small	Death
Aeolosoma	SD JP-4	D, FT	25	450 TC	2	small	Death
Daphnia	PJP-4	D, SR	20	none	2	<24 h old	Death
Daphnia	PJP-4	D, FT	22	none	2	<24 h old	Death
Daphnia	SD JP-4	D, SR	20	none	2	<24 h old	Death
Daphnia	SD JP-4	D, FT	22	none	2	<24 h old	Death
Paratanytarsus	PJP-4	D, FT	27	monolayer S	2	3rd instar	Death
Paratanytarsus	SD JP-4	D, FT	22	monolayer S	2	3rd instar	Death
Microbial Communities	PJP-4	D, FT	24	none	2	mature, colonized	Species Elimination
Microbial Communities	SD JP-4	D, FT	23	none	2	mature, colonized	Species Elimination

[1] D=definitive, P=preliminary, SR=static-renewal, FT=flow-through

[2] TC=trout chow suspension, S=builders sand

response, an EC20, the concentration necessary to produce a 20% change in response relative to the mean control value, was estimated from the regression line by means of inverse prediction.

C. Toxicity Tests With Microbial Communities

The ability of naturally derived microbial communities to maintain their structural integrity and diversity under acute toxic stress was examined by exposing colonized artificial substrates to jet fuel WSFs for 48 h. Polyurethane foam (PF) substrates (3.75 X 5.0 X 3.0 cm) were suspended in a nearby, unpolluted, montane pond (Pandapas Pond, Jefferson National Forest, Montgomery County, VA) and allowed to colonize until a relatively constant number of protozoan species was obtained. Previous research has indicated that an equilibrium number of 40 to 60 species is reached within 10 to 14 d and maintained thereafter (Pratt 1984). These colonized PF substrates were retrieved and transported to the laboratory. Three PF substrates were examined immediately to determine the number of resident protozoan species. Additional substrates were exposed to WSFs for 48 h in the modified Mount-Brungs dilutor described previously; then the number of protozoan species remaining was determined. Each substrate was sampled by squeezing it over a 500-ml beaker. The number of protozoan species was determined within 12 h of sampling by pipetting two drops of

material from the bottom of the beaker onto microscope slides, covering with a No. 1, 22 mm coverglass, and scanning systematically at 200x or 450x total magnification. Generally, two subsamples from each beaker were examined. However, if the second slide increased the total species number by more than 30%, a third slide was examined. Protozoans were identified to genus and to species whenever possible using standard taxonomic keys (e.g., Kahl 1930-1935, Kudo 1971, Page 1976, Pascher 1913-1927). Species occurrence was recorded on a master list. Differences in species numbers on exposed and control substrates were examined with ANOVA and Duncan's multiple range tests.

Chronic toxicities of WSFs to microbial communities were evaluated by monitoring the colonization of barren PF substrates under toxic stress. Colonization is the means by which microbial communities perpetuate themselves and as such is analogous to reproductive success in single species toxicity tests. After preliminary tests in the Mount-Brungs dilutor, definitive chronic tests were conducted in the system shown in Figure 23. The WSFs were manufactured and diluted as previously described, but higher volumes were accomodated by delivering a 225-ml volume from the diluter into 1-l central beakers every 3 min. The toxicant was then pumped to the mixing chambers and diluted 1:1 with "upstream" water. The "upstream" water consisted of

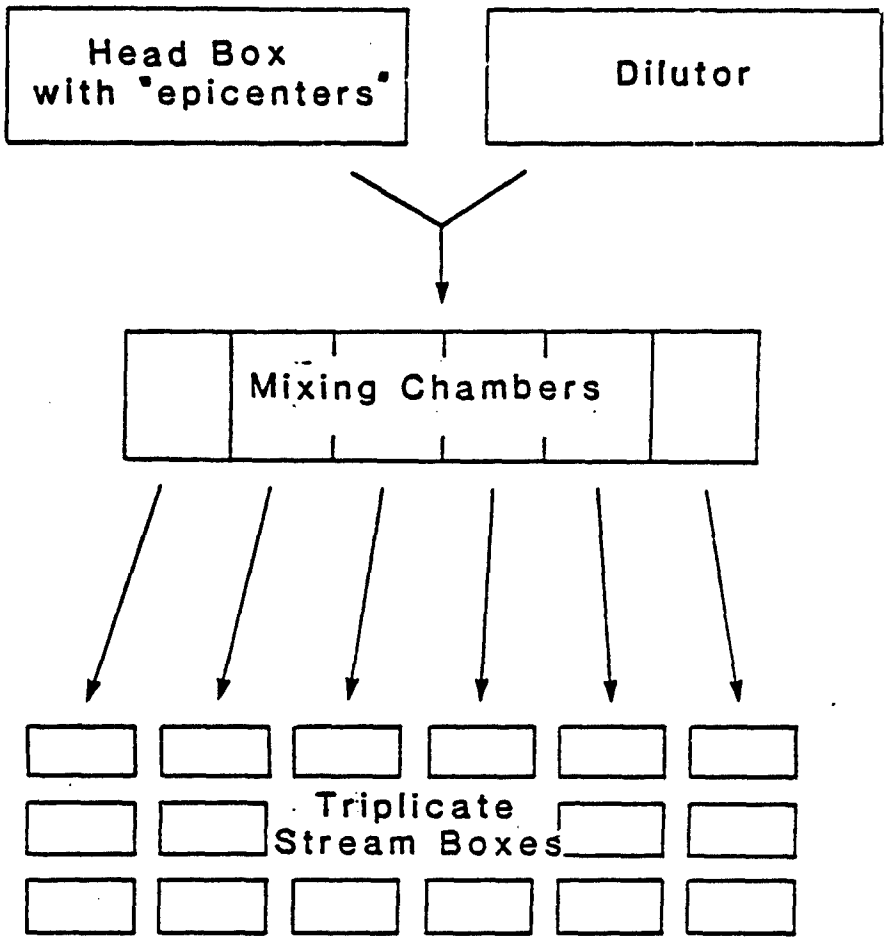

Figure 23: Schematic of the flow-through test system used to test response of microbial communities to toxicant stress. Epicenters are placed in the head box, water and test compound from the Mount-Brungs dilutor mix in chambers, then enter stream boxes containing uncolonized substrates.

dechlorinated tap water that was passed through a headbox containing "epicenter" communities, i.e., PF substrates that had been colonized to equilibrium : · Pandapas Pond. The "upstream" headbox was seeded with one colonized, "epicenter" PF substrate for each test chamber (12 to 18 total). This water served as the source of organisms for the colonization of barren substrates in the test chambers. From the mixing chambers, the test material was delivered to three replicate stream boxes (46 X 31 X 15 cm) containing barren PF substrates and 10 l of water. Test material was siphoned out of the stream boxes on the side opposite entry. Rates were adjusted throughout to deliver 40 to 50 ml min^{-1} to each stream box. This system provided a replacement volume of 10 l every 4 h. Light for the headbox and stream boxes was provided by Durotest Vitalite fluorescent bulbs at an intensity of 500 ft-c at the air-water interface on a photoperiod of 16 h light and 8 h dark.

Four barren (i.e., uncolonized) PF substrates (3.75 X 2.5 X 3.0 cm) were suspended in each stream box. One substrate was removed from each stream box at intervals over 10 d. The number of protozoan species occurring on each substrate was determined as described above. Colonization curves describing the accrual of species on the barren substrates over time according to the MacArthur-Wilson model of noninteractive colonization (MacArthur and Wilson 1967) were fitted and compared. The model used was:

$$S_t = S_{eq} (1-e^{-Gt})$$

where S_t is the number of species on day t, S_{eq} is the asymptotic or equilibrium number of species, and G is the growth or colonization rate. Nonlinear regression using "dummy" variables to identify treatment groups was used to fit and compare all colonization curves to that for control streams (e.g., Kleinbaum and Kupper 1978). In addition, chlorophyll *a* and adenosine triphosphate (ATP) measurements were made on the samples on day 7. Triplicate chlorophyll *a* determinations were made on each sampled substrate using the phaeophytin corrected fluorometric method (APHA *et al.* 1981). A 1-ml sample was injected into 9 ml 100% spectrograde acetone. This 90% mixture was extracted at 4°C for 24 h in the dark. Measurements were made immediately after extraction using a Turner Designs fluorometer. Similarly, three subsamples of the substrate contents were extra:ed for ATP analyses. Each 1-ml sample was injected into 9 ml boiling tris buffer (tris (hydroxymethyl) aminomethane, 0.02 M, pH 7.75) and heated in a boiling water bath for 5 min. The extractions were frozen at -20°C until analysis. The ATP measurements were made using the luciferin-luciferase bioluminescence technique (APHA *et al.* 1981). A Labline 9140 ATP photometer and Firelight Enzyme (Analytical Luminescence Lab Inc.) were used. An

autotrophic index (AI) was then calculated for each PF substrate from mean chlorophyll *a* and ATP values as follows:

$$AI = \frac{\text{ATP biomass (mg/l)}}{\text{chlorophyll } a \text{ (mg/l)}}$$

where:

$$\text{ATP biomass (mg/l)} = \frac{\text{ATP (ng/l)}}{\text{2,400 (mg biomass/ng ATP)}}.$$

Differences in chlorophyll *a* content, ATP biomass, and AI were examined with ANOVA. Duncan's multiple range test was used to locate differences in balanced tests. Scheffe's multiple comparison test was used for tests with unequal sample sizes.

III. RESULTS AND DISCUSSION

A. Toxicant Concentrations

As we reported in the Second Annual Report, longer tests had substantial problems with declines in benzene and toluene concentrations in test vessels. Continuously prepared WSFs were less concentrated than the Standard Maximal WSF, ranging from 5.5 to 48.7% MaxWSF. Lower concentrations were expected since the on-line production of WSF occurred under

less favorable conditions than those in which the standards were prepared. However, even with constant input and turnover, benzene and toluene concentrations in the test vessels steadily declined over time, often to <10% of the levels obtained at the start of the test. This loss over time was probably due to increasing populations of bacteria that degraded jet fuel components. Evidence for biodegradation comes from several observations. Casual observation of both the fractionators and test chambers during long-term tests showed increasing turbidity with time and toxicant concentration. This casual observation of increased bacterial growth in higher WSF concentrations was experimentally confirmed in one chronic test with biomass estimates from ATP measurements. On day 17 of the chronic exposure of Daphnia pulex to WSF P JP-4, ATP determinations were made on water samples as delivered to test vessels (see Part III, Section II.C for methods). The ATP biomass and, presumably, microbial density of test solutions increased regularly with increases in toxicant concentrations (Figure 24).

Several approaches to controlling populations of bacteria were considered but judged inappropriate. Ultraviolet (UV) light is often used in aquarium systems to control bacterial growth. However, it has been found that the toxicity of some organic wastes can be strongly affected by UV light (Allred and Giesey 1983). Antibiotics might control

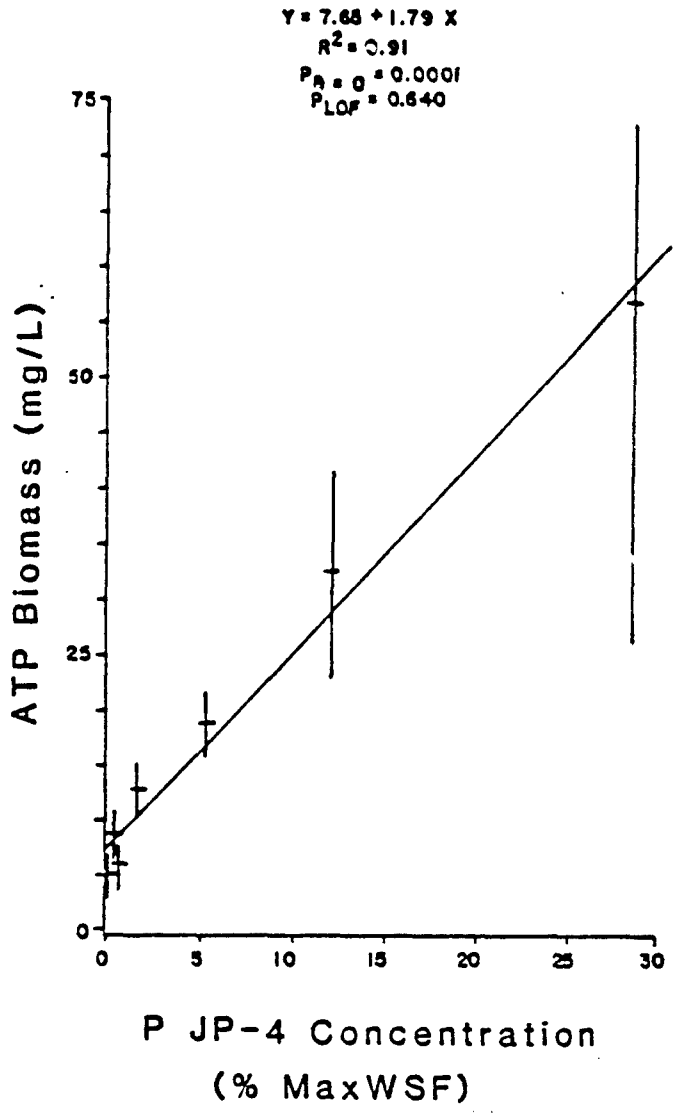

Figure 24: Relationship between ATP biomass and WSF P JP-4 concentrations in test water. Data collected on day 17 of chronic test with Daphnia pulex. Hatch marks are means. Bars span two standard deviations.

bacterial growth, and controls with and without antibiotics could be compared. However, documenting synergistic or antagonistic effects between jet fuel components and antibiotics would be difficult, particulary in community level tests.

Within the scope of the current project, the most practical approach to problems with biodegradation and volatilization was to maintain exposures at the highest doses continuously attainable in the flow-through exposures and document declines in benzene and toluene. The only attempt to control bacterial growth was a weekly cleaning of those parts of the exposure systems that could be cleaned without disrupting exposures. Concentrations are reported as the geometric mean of measured concentrations (% MaxWSF, as previously described) followed parenthetically by the range of concentrations observed over the length of the test. Although benzene and toluene exposure levels decreased over time, organisms continued to be exposed to some jet fuel components and/or their breakdown products. Similar degradation would be expected in the field and would certainly reduce the severity of any deleterious effects on aquatic systems from jet fuel exposure. Specific fate studies could document these processes and help determine environmentally realistic exposure levels.

B. Invertebrate Toxicity Tests

1. Aeolosoma

Acute exposures to concentrations of WSF P JP-4 ≤8.9% MaxWSF were not lethal to A. headleyi. Exposure to 20.3% (19.9-20.7) MaxWSF, the highest concentration attained, was lethal to only 17% of the organisms tested. Thus, no LC50 could be determined from this test.

Chronic exposures to WSF P JP-4 resulted in significant decreases in the population growth of A. headleyi ($p = 0.0006$, Figure 25). The LOEC was 8.9%; NOEC, 1.2%; and MATC, 5.1%. There was a significant relationship between WSF P JP-4 concentrations and the number of worms at the end of the test (Figure 26). The EC20 estimated from this relationship was 7.0% MaxWSF (CL = 1.3 to 13.3% WSF).

Acute exposure to 26.3% (25.6-27.0) MaxWSF, the highest concentration of WSF SD JP-4 attained, was not lethal to A. headleyi. Survival in this concentration was 96.7%.

In chronic WSF SD JP-4 exposures, 26.3% (8.5-27.0) MaxWSF had no significant effect on the population growth of A. headleyi. There were no significant differences in the number of worms present after 7 d (Figure 27, $p = 0.438$).

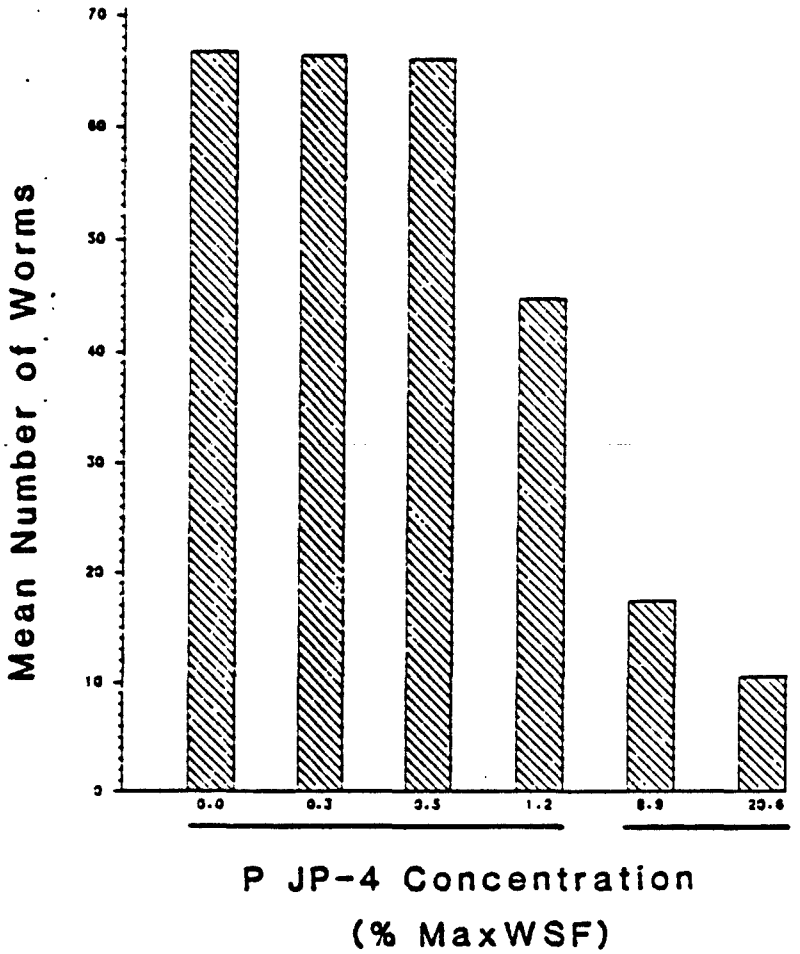

Figure 25: Effects of chronic WSF P JP-4 exposure on population growth of Aeolosoma headleyi. Bars connected by the same line are not significantly different at the 0.05 level.

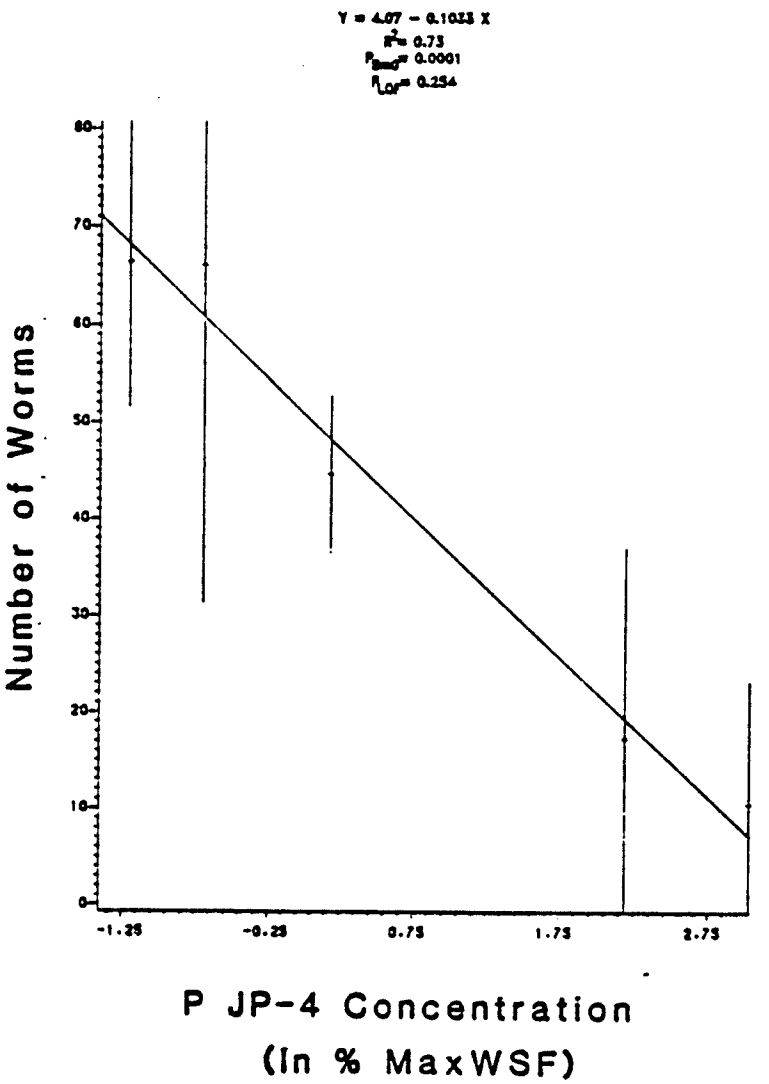

Figure 26: Relationship between population growth of Aeolosoma headleyi and WSF P JP-4 concentrations. Hatchmarks are means. Bars span two standard deviations.

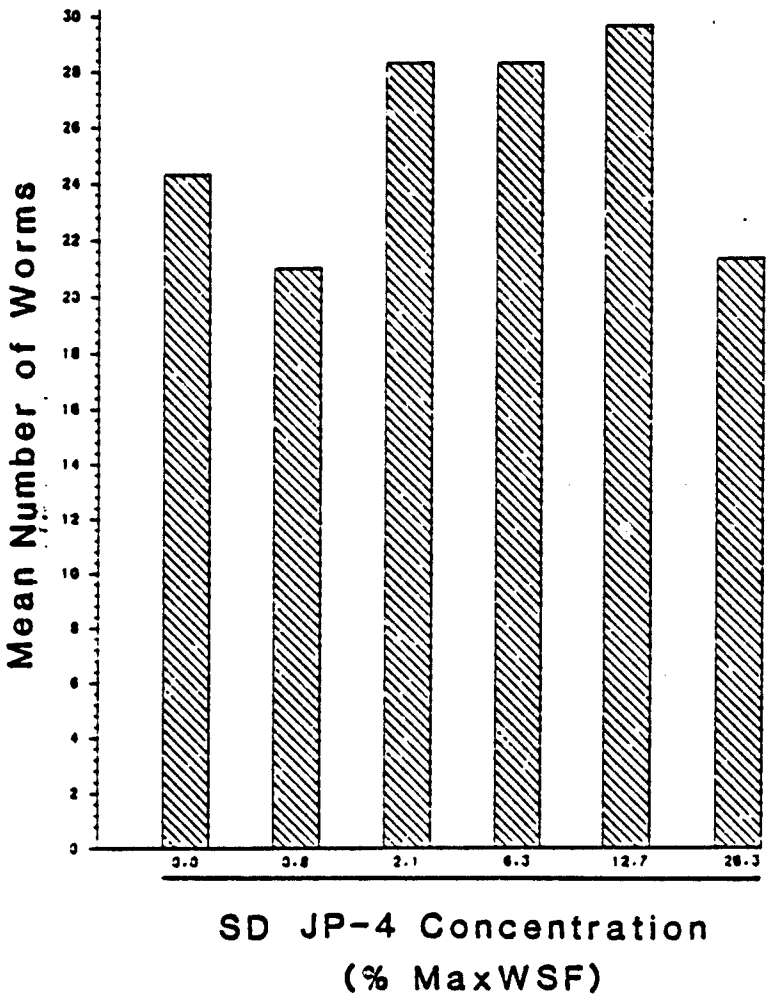

Figure 27: Effects of chronic WSF SD JP-4 exposure on population growth of Aeolosoma headleyi. Bars connected by the same line are not significantly different at the 0.05 level.

2. Daphnia

Two acute toxicity tests were conducted exposing D. pulex to WSF P JP-4. The first test was static with renewal of the test solutions every 24 h. There was 73% survival in the highest concentration attained. In this test, the undiluted WSF was 33.7% MaxWSF at set up and renewal and declined to 17.2% after 24 h, averaging 25.5%. In a flow-through test, the highest concentration attained was 19.6% (18.4-20.9) MaxWSF with 100% survival in this concentration.

A 21-d exposure to WSF P JP-4 resulted in significant effects on survival, growth, and reproduction of D. pulex (Table 12). Survival was affected at measured concentrations ≥11.9% MaxWSF (LOEC = 11.9%, NOEC = 5.2%, and MATC = 7.9%). Growth after 7 d was also affected at 11.9%. Although the relationship between dose and length on day 7 was significant ($p = 0.0008$), the coefficient of determination was quite low ($r^2 = 0.15$); consequently, no EC20 was estimated from this relationship. Reproduction was significantly different from the control in all exposure concentrations. However, the relationship between dose and reproductive success was not a simple one. At low and high doses of WSF P JP-4, reproduction was inhibited, but at intermediate doses there was stimulation. This pattern of response may be an example of a substance providing both a subsidy and a stress to aquatic organisms (e.g., Odum et al.

TABLE 12

Effects of chronic WSF PJP-4 exposure on Daphnia pulex.
Concentrations are reported as geometric means and ranges.
Organism responses are reported as means ±1 standard deviation

Nominal Concentration (%)	Mean Measured Concentration (% MaxWSF)	21 d Survival (Alive/Total)	7 d Growth (Length in mm)	21 d Reproduction (Young per vessel)
Control	BD[a]	13/15	1.53 ±0.11	108.3 ±14.0
3	0.4 (0.2-0.8)	10/15	1.49 ±0.11	75.7[b] ±20.6
6	0.6 (0.3-1.2)	12/15	1.43 ±0.11	40.7[b] ± 9.0
12	1.6 (0.9-3.0)	13/15	1.50 ±0.11	158.7[b] ±22.6
25	5.2 (2.8-15.9)	14/15	1.60 ±0.14	247.7[b] ±22.1
50	11.9 (7.6-24.7)	7/15[b]	1.27[b] ±0.14	66.7[b] ± 5.1
100	28.5 (18.2-43.1)	0/15[b]	---- ----	0.0[b] ---

[a]BD = below detection
[b]Significantly different from the control at the 0.05 level

1979). Jet fuel WSF encouraged the growth of bacteria (see Part III, Section III.A), which are a high quality food resource for D. pulex (e.g., Mount and Norberg 1984). At moderate exposure levels, this increase in food supply may have increased reproductive success. However, at higher concentrations, the toxic effects of jet fuel WSF outweigh the food advantage. At concentrations of 11.9% MaxWSF, reproductive success is reduced to levels below that of the controls. This concentration corresponds to the first exposure level also affecting both survival and growth.

Static-renewal and flow-through acute toxicity tests were conducted with WSF SD JP-4. In the static-renewal test the highest concentration attained was 29.6 % MaxWSF at set up and 0.8% MaxWSF after 24 h, averaging 6.1%. Survival of D. pulex in this concentration was 100%. In a flow-through exposure the highest concentration attained was 16.8% (16.7-16.8) MaxWSF and there was 100% survival at this concentration.

There were significant effects on survival, growth, and reproduction of D. pulex in a 21-d exposure to WSF SD JP-4 (Table 13). Survival was affected at concentrations ≥ 1.1% MaxWSF (LOEC = 1.1%, NOEC = 0.1%, and MATC = 1.8%). The SD JP-4 exposed daphnids were significantly smaller than controls in concentrations ≥2.9% (LOEC = 2.9, NOEC = 1.1, MATC = 1.8%). Reproductive success was decreased at

TABLE 13

Effects of chronic WSF SD JP-4 exposure on Daphnia pulex.
Concentrations are reported as geometric means and ranges.
Organism responses are reported as means ±1 standard deviation

Nominal Concentration (%)	Mean Measured Concentration (% MaxWSF)	21 d Survival (Alive/Total)	7 d Growth (Length in mm)	21 d Reproduction (Young per vessel)
Control	BD[a]	13/15	1.89 ±0.08	405.3 ±37.4
3	0.1 (BD[a]-0.2)	14/15	1.74 ±0.07	372.0 ±56.3
6	--Mechanical failure. Concentration eliminated.--			
12	1.1 (0.1-1.6)	1/15[a]	1.87 ±0.19	210.3[a] ±89.5
25	2.9 (0.5-4.5)	0/15[a]	1.72[a] ±0.10	155.7[a] ±50.4
50	8.9 (1.1-10.9)	0/15[a]	1.67[a] ±0.08	61.0[a] ±13.5
100	25.2 (3.2-29.7)	0/15[a]	1.32[a] ±0.10	9.0[a] ±15.6

[a]BD = below detection
[a]Significantly different from the control at the 0.05 level

concentrations ≥1.1%. The number of young produced in each test vessel declined with increasing concentration, and the relationship between dose and response was significant (Figure 28). The EC20 estimated from this regression line was 0.20% (CL = 0.04-0.50). Reproduction in control groups was higher than that observed in the P JP-4 test, probably due to increased feeding rate (see Table 11), and no toxicant related subsidy was observed in growth or reproductive success.

3. Paratanytarsus

There were significant effects of WSF P JP-4 on the survival of third instar P. parthenogenica after a 48-h exposure. Concentrations ≥0.7% MaxWSF significantly reduced survival, and, in the highest concentration attained, 40.3% (34.3-49.7) MaxWSF, there was no survival after 48 h. The probit estimate of the 48-h LC50 was 2.2% (FL = 1.6-2.9%) MaxWSF. The slope of the dose-response line was 1.85 (CL = 1.59-2.11).

In a chronic test, there were significant effects of WSF P JP-4 on the survival P. parthenogenica at concentrations ≥1.4% MaxWSF (LOEC = 1.4%, Table 14). The NOEC was 0.6% MaxWSF, and the MATC was 0.9%. There were also significant effects on larval size after 7 d. Larvae in 0.6% MaxWSF were significantly larger than controls, suggesting a

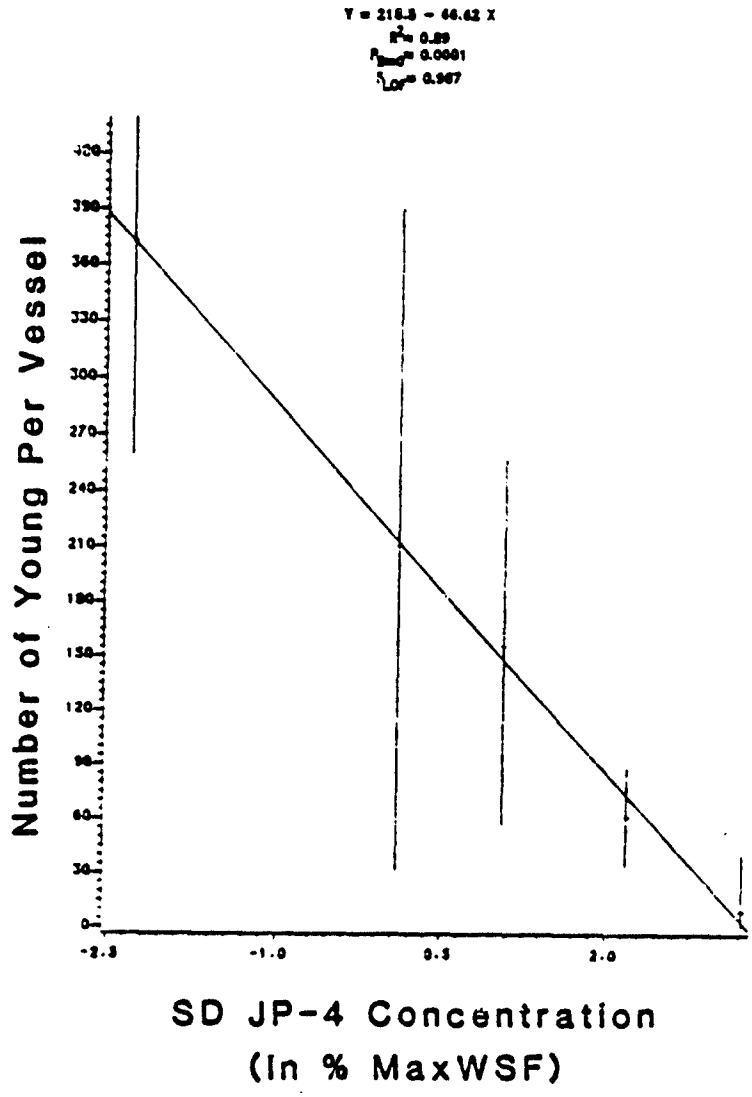

Figure 28: Relationship between reproductive success of Daphnia pulex and WSF SD JP-4 concentrations. Hatch marks are means. Bars span two standard deviations.

TABLE 14

Effects of chronic WSF P JP-4 exposure on *Paratanytarsus parthenogenica*. Concentrations are reported as geometric means and ranges. Organism responses are reported as means ±1 standard deviation

Nominal Concentration (%)	Mean Measured Concentration (% MaxWSF)	21 d Survival (Alive/Total)	7 d Growth (Length in mm)	21 d Reproduction (Eggs per vessel)
Control	BD[a]	27/51	1.27 ±0.15	431.7 ±277.5
3	0.2 (BD[a]-0.4)	31/50	1.14[b] ±0.09	624.7 ±157.0
6	0.3 (0.1-0.9)	35/56	1.25 ±0.13	1080.7[b] ± 43.2
12	0.6 (0.1-1.8)	29/52	1.45[b] ±0.19	614.7 ±492.2
25	1.4 (0.3-3.6)	14/55[b]	1.38 ±0.24	305.3 ±275.5
50	3.8 (1.4-9.6)	13/49[b]	1.13 ±0.06	55.0 ± 95.3
100	6.3 (1.6-16.1)	0/51[b]	0.75[b] ±0.13	0.0[b] ---

[a]BD = below detection
[b]Significantly different from the control at the 0.05 level

possible bacterial food subsidy. Larvae in 0.2 and 6.3% MaxWSF were significantly smaller than controls. The difference in the 0.2% exposure group was unexpected and may be anomalous, since there were no corresponding differences from the control in the other two measures of chronic toxicity, i.e., survival or reproductive success. Although reproductive success declined at higher doses, egg production in each concentration was quite variable. The only significant difference from the control was a stimulation of reproduction at 0.6%. Again, this may be due to a food subsidy related to increased bacterial growth in jet fuel exposures.

Survival to the end of the test in control vessels was 53%. This value is substantially below the 80% survival rate considered a minimum in chronic tests of similar length with Daphnia (e.g., OECD 1981). Low control survival suggests that culture and test methods require further development. Problems with chironomid tests may include sensitivity to turbulence in early instars (Anderson 1980), inability to retain the small early life stages in flow-through test vessels, and inadequate nutrition. Our difficulties with chironomid survival are not unique. Similar, low survival rates have been reported previously (e.g., Lee et al. 1980).

In an acute exposure to WSF SD JP-4, 17.4% (16.4-38.0) MaxWSF, the highest concentration attained, was lethal to

20% of third instar P. parthenogenica tested. No LC50 could be calculated for this test.

Flow-through chronic tests exposing chironomids to WSF SD JP-4 were set up on three occasions, but in each case, tests were terminated due to poor control group hatching, survival, or retention. A final attempt to obtain chronic effect data with this species was made using a static-renewal system. After a 4-d exposure, hatching was completely inhibited at the highest concentration attained, 23.0% MaxWSF at set up, declining to 0.1% after 24 h, averaging 1.7%. Hatching was unaffected at the next concentration tested, 0.3% (0.1-5.8) MaxWSF. The LOEC was 1.6% MaxWSF; NOEC, 0.3%; and MATC, 0.7%.

C. Microbial Community Toxicity Tests

There were no significant differences in the number of protozoan species remaining on PF substrates after a 2-d exposure to WSF P JP-4. The highest concentration attained, 35.9% (27.8-48.9) MaxWSF, had 14% fewer species than control substrates. The mean number of protozoan species on control substrates was 48.7 (SD±3.1), and there were 42.0 (SD±6.0) species on substrates exposed to 35.9% WSF P JP-4.

In chronic exposures to WSF P JP-4, 0.09% (0.01-2.00) MaxWSF, the highest concentration attained, had no

discernible effect on the colonization of barren substrates (Figure 29). There were no differences in either the rate of colonization or in the number of protozoan species at equilibrium (Table 15). However, there were differences in the structure of communities in chlorophyll a content and in their AI after 7 d. Higher jet fuel exposures tended to have less chlorophyll a. Only the highest exposure concentration had AI values significantly different from the control. However, all exposed substrates had higher AI values than controls, and all exposed substrates exceeded a value of 200. An AI of 200 is considered an indication of pollution stress (APHA et al. 1981) and is characteristic of organic pollution.

Final concentrations in this test were low despite continuous input and renewal of WSF P JP-4. The test system produced WSF at 8.5% (5.5-10.8) MaxWSF. Expected concentrations ranged from 3 to 50% of this amount, 0.3 to 4.3 % MaxWSF. However, after the first 2 d, concentrations in test vessels did not exceed 0.1% in any vessel. Although toxicant was completely replaced approximately five times in 24 h, this flow rate was apparently insufficient to maintain higher concentrations. The substrate and "upstream" water provided for the development of microbial communities may have encouraged the growth of bacteria capable of degrading jet fuel components. Because concentrations declined over

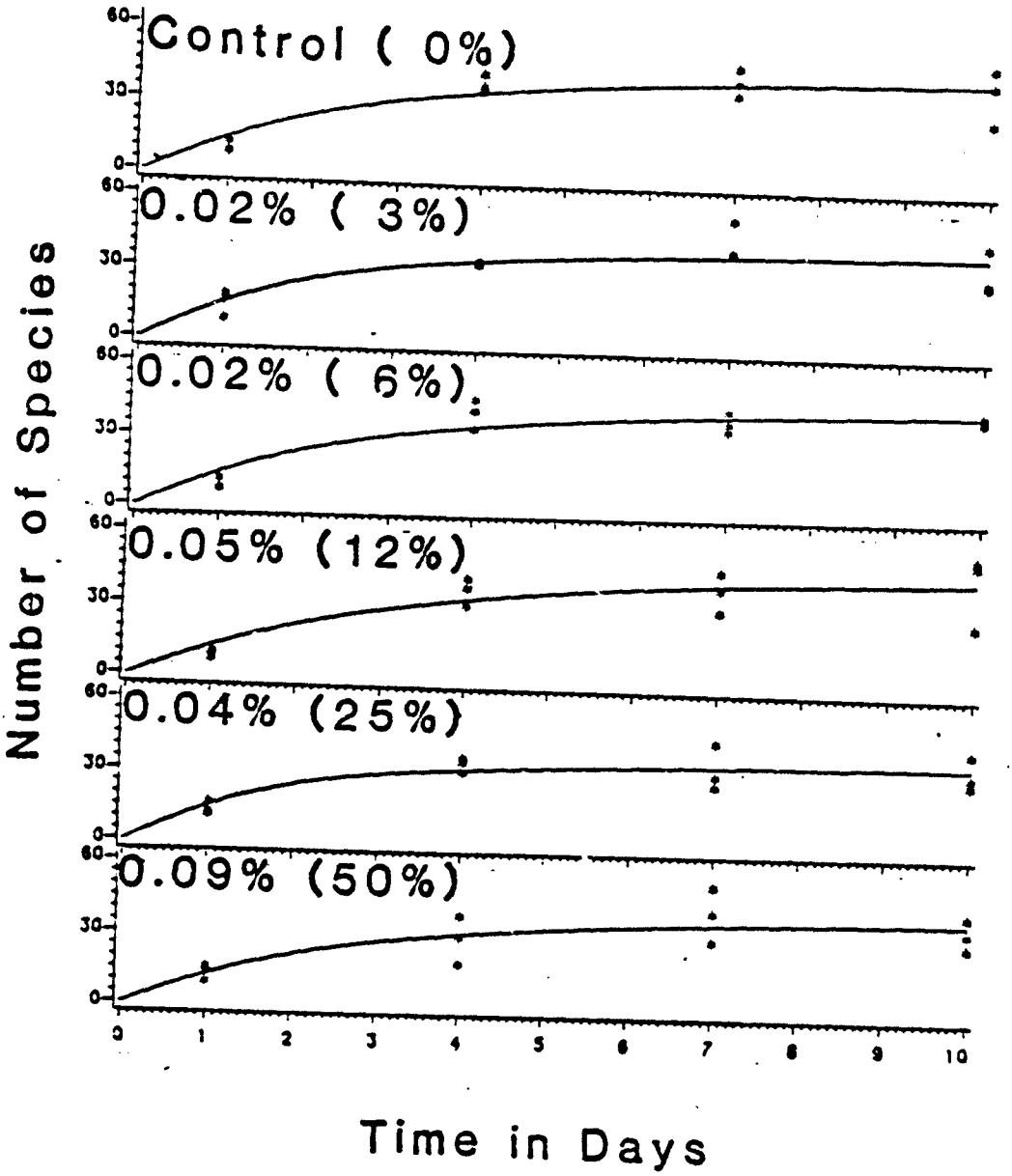

Figure 29: Effects of chronic WSF P JP-4 exposure on colonization of barren substrates by protozoans. Asterisks are observed numbers of species. Lines are fitted colonization curves. Measured concentrations (% MaxWSF) are followed by nominal concentrations in parentheses.

TABLE 15

Effects of chronic WSF P JP-4 exposure on microbial communities. Concentrations are reported as geometric means and ranges. Community responses are reported as means ±1 standard deviation

Nominal Concentration (%)	Mean Measured Concentration (% MaxWSF)	Seq	G	ATP Biomass	Chlorphyll a	AI
Control	BD[a]	42.8 ±4.0	0.40 ±0.13	72.8 ±17.1	0.46 ±0.15	166.6 ± 21.4
3	0.02 (≤0.01-0.21)	39.1 ±3.5	0.49 ±0.17	61.5 ±25.5	0.17[b] ±0.09	443.8 ±325.2
6	0.02 (≤0.01-0.21)	42.2 ±3.3	0.40 ±0.11	117.3 ±27.9	0.54 ±0.18	235.2 ± 95.9
12	0.05 (≤0.01-0.34)	46.3 ±6.5	0.32 ±0.13	102.4 ±26.6	0.37 ±0.04	281.0 ± 80.1
25	0.04 (≤0.01-0.60)	34.5 ±2.6	0.56 ±0.17	82.0 ±11.3	0.23[b] ±0.05	370.4 ± 41.3
50	0.09 (0.01-2.00)	38.3 ±4.9	0.39 ±0.17	109.5 ±35.9	0.21[b] ±0.03	527.8[b] ±166.2

[a]BD = below detection
[b]Significantly different from the control at the 0.05 level

time, a time dependent process, such as the growth of biodegrading bacteria, is most likely to be a major cause.

There were no significant differences in the number of protozoan species remaining on naturally colonized PF substrates after a 2-d exposure to WSF SD JP-4. The number of species on control substrates and those exposed to 14.6% (6.1-11.4) MaxWSF, the highest concentration attained, were 48.7 (SD±1.5) and 47.3 (SD±4.2), respectively.

In chronic tests with WSF SD JP-4, exposure to 0.20% MaxWSF, the highest concentration obtained, had no discernible effect on the colonization of barren substrates by protozoans (Table 16 and Figure 30). There were no significant differences between exposure groups in colonization rate or equilibrium species number ($p = 0.061$). There were significant differences between groups in ATP biomass and in chlorophyll *a* content of the sustrates on day 7. Control substrates had the lowest ATP biomass, the highest chlorophyll *a* content, and an AI below 200. Only the highest exposure group, 0.20% MaxWSF, was consistently different from the control in these measures.

TABLE 16

Effects of chronic WSF SD JP-4 exposure on microbial communities. Concentrations are reported as geometric means and ranges. Community responses are reported as means ±1 standard deviation

Nominal Concentration (%)	Mean Measured Concentration (% MaxWSF)	Seq	G	ATP Biomass	Chlorphyll a	AI
Control	BD[a]	42.8 ±4.0	0.40 ±0.13	72.8 ±17.1	0.46 ±0.15	166.6 ± 21.4
3	0.07 (0.06-0.22)	45.9 ±17.8	0.08 ±0.04	70.8[b] ±18.0	0.30 ±0.24	539.9 ±574.2
12	0.10 (0.05-0.67)	41.6 ±11.2	0.11 ±0.05	53.7 ± 6.5	0.34 ±0.06	162.8 ± 35.4
50	0.20 (0.03-3.60)	42.9 ±16.4	0.11 ±0.07	73.6[b] ±19.5	0.10[b] ±0.05	807.7[b] ±304.9

[a]BD = below detection
[b]Significantly different from the control at the 0.05 level

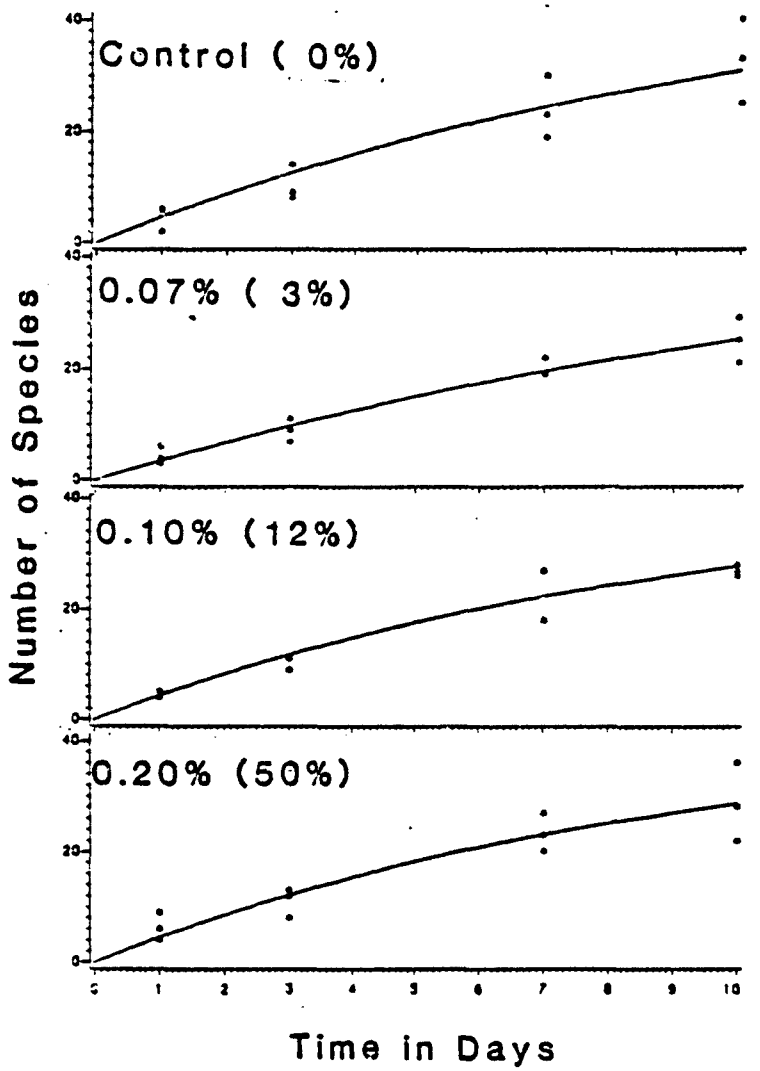

Figure 30: Effects of chronic WSF SD JP-4 exposure on colonization of barren substrates by protozoans. Asterisks are observed numbers of species. Lines are fitted colonization curves. Measured concentrations (% MaxWSF) are followed by nominal concentrations in parentheses.

IV. COMPARISONS AND CONCLUSIONS

Tables 17 and 18 summarize the acute and chronic effect data obtained in this study. A number of trends can be identified. With the exception of Paratanytarsus exposed to WSF P JP-4, neither jet fuel WSF was particularly toxic to invertebrates or microbial communities in acute exposure. The low acute toxicity to invertebrates and the greater toxicity to fish are consistent with data on individual toxicities of benzene and toluene, the major components of the WSFs. Benzene concentrations in tested WSFs were ≤6 mg/l, yet the Water Quality Criterion Document for benzene indicates that concentrations of 203 to 620 mg/l are neccessary to obtain an acute effect on daphnids (USEPA 1980a). Fish were considerably more sensitive, and effect levels ranged from 5 to 386 mg/l. Toluene concentrations did not exceed 4 mg/l in invertebrate exposures. Other data suggest that 60 to 313 mg/l are required to obtain an LC50 with D. magna (USEPA 1980b). Again, fish are more sensitive as effect levels ranged from 13 to 59 mg/l. The low acute toxicity of both WSFs precluded a direct comparison of the toxicities of P JP-4 and SD JP-4.

It is possible that the midge, Paratanytarsus, is more sensitive to WSF P JP-4 than other tested organisms. However, it is also possible that the 48-h LC50 for Paratanytarsus exposed to WSF P JP-4 is a less reliable

TABLE 17

Summary of acute effect levels for jet fuel WSFs.
Values are LC50s from acute tests. Concentrations are % MaxWSF

Test Organism	P JP-4 WSF	SD JP-4 WSF
Aeolosoma	>20.6	>26.3
Daphnia	>25.5	>16.8
Paratanytarsus	2.2	>17.4
Microbial Communities	>35.9	>14.6
Lepomis	26.2	23.3

TABLE 18

Summary of chronic effect levels for jet fuel WSFs.
Values are LOECs from chronic tests. Concentrations are % MaxWSF

Test Organism	P JP-4 WSF	SD JP-4 WSF
Aeolosoma	8.9	>26.3
Daphnia	0.3, 11.9	1.1
Paratanytarsus	1.7	1.6
Microbial Communities	0.09	0.20
Lepomis		
21 d test	>0.42	0.95
Ventilation	5.1	1.5
Preference-Avoidance	3.5	>14.0
Blood Chemistry	13.0	>22.0
Histology	13.0	22.0

index of acute effects. Our difficulties in keeping control organisms alive in later chronic tests suggest deficiencies in methods of culturing and testing this organism.

Interpretation of all chronic test results was complicated by toxicant losses over time. These losses occurred despite continuous input and renewal. The consistency and magnitude of toxicant losses suggest that both volatilization and biodegradation would be significant factors in the field and would ameliorate any deleterious effects of jet fuel releases.

Chronic tests suggest moderate toxicity of jet fuel WSFs to aquatic organisms. The LOECs for WSF P JP-4 ranged from 0.09% MaxWSF for microbial communities to 8.9% MaxWSF for A. headleyi. The LOECs for WSF SD JP-4 ranged from 0.20% MaxWSF for microbial communities to ≥26.3% MaxWSF for A. headleyi. Similar ranges of chronic effect levels, i.e., across two orders of magnitude, are not uncommon. There was no noticable dichotomy in chronic sensitivity between fish and invertebrates. Very little data exists on the effects of chronic exposures of benzene or toluene to aquatic organisms for comparison.

Of the toxicity tests used, microbial communities appeared most sensitive to exposure to jet fuel WSFs. However, the observed shifts to heterotrophy would probably be short

lived after inputs were curtailed. Other community level changes, e.g., reductions in diversity, were not observed at such low concentrations. Fish ventilatory rates provided chronic effects data in the shortest time and provided a reasonable indication of chronic effect levels for other organisms tested.

PART IV

I. ADMINISTRATIVE

A. Personnel

Dr. John Cairns, Jr., University Distinguished Professor, Department of Biology, and Director, University Center for Environmental Studies, served as Principal Investigator of this project. Dr. Arthur L. Buikema, Jr., Professor of Zoology, Department of Biology, served as Senior Investigator for this project.

The primary researcher on this project was Major Thomas R. Doane, a graduate student and research associate completing work on a Ph.D. in Zoology in the Department of Biology, Virginia Polytechnic Institute and State University. He started his degree work and work on this project in July 1981. His education was financed by the Air Force Institute of Technology (AFIT). He did not receive any financial assistance from this grant.

A full-time Laboratory Specialist, Ms. Barbara R. Niederlehner, and a full-time Laboratory Technician, Ms. Sylvia Sanford, worked on this project.

Other part-time personnel contributing to this project were Dr. David Stetler, Associate Professor of Botany, and Mr. George Schupin, who did much of the electron microscopy, although not a part of the supported personnel. Miss Rebecca Allen provided secretarial support. There have also been several other individuals who have assisted and been paid on an hourly basis for laboratory and secretarial support. Among these are Janet Webster and Margaret Chang. Mr. Brewer Pedin helped with much of the instrumentation.

B. Presentations

Major Doane presented a paper at the Virginia Academy of Science on the results of sublethal effects of WSF P JP-4 exposure on bluegill in May 1983.

Major Doane also presented a poster session at the Society of Environmental Chemistry and Toxicology in November 1983. This poster presented the data on the bluegill research up to that date.

Major Doane presented a paper at a specialized conference held in Cardiff, UK, in September 1984. This presentation has been published as follows:

Doane, T. R., Cairns, John, Jr., and Buikema, Arthur L., Jr. (1984). Comparison of biomonitoring techniques for evaluating effects of jet fuel on bluegill sunfish (Lepomis macrochirus). In: Advances in Water Pollution Control--Freshwater Biological Monitoring. D. Pascoe, and R. W. Edwards (Eds.). Pergamon Press, New York, NY. pp. 103-112.

II. RESEARCH OBJECTIVES

Original research objectives were:

1. Determine the acute and chronic toxicity of the water soluble fraction of a jet fuel (WSF JP-4) to the bluegill sunfish (Lepomis macrochirus) after conventional and episodic dosing.

2. Determine the effects of sublethal concentrations of WSF JP-4 on fish ventilation rates.

3. Determine the effects of sublethal concentrations of WSF JP-4 on fish preference-avoidance behavior.

4. Determine the effects of sublethal concentrations of WSF JP-4 on fish blood chemistry.

5. Determine the effects of sublethal concentrations of WSF JP-4 on selected fish tissues.

6. Determine the acute and sublethal effects of WSF JP-4 on selected aquatic invertebrates.

7. Determine the effects of WSF JP-4 on microbial communities.

8. Compare data collected for the objectives outlined above and evaluate capabilities for predicting sublethal effects from the parameters measured.

Original research objectives were met.

III. BUDGET

The financial report will be submitted by the Virginia Tech Office of Funded Research.

IV. QUESTIONS

If there are any questions on this report please contact:

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